ADHESION MOLECULE EXPRESSION AND CELLULAR INFILTRATE WITHIN GINGIVAL TISSUE

By

Naureen Arshad Moughal B.Sc. (Hons) (Glasg)

Thesis submitted for the degree of Doctor of Philosophy to the Faculty of Medicine, University of Glasgow

Unit of Periodontology, Department of Adult Dental Care, University of Glasgow Dental School

Department of Immunology, Western Infirmary, University of Glasgow

To my parents and my family

CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

LIST OF TABLES

CHAPTER 2

CHAPTER 3

ABBREVIATIONS

ABC	avidin-biotin complex
ABC-AP	ABC-alkaline phosphatase kit
ABC-P	ABC-peroxidase kit
AP	adult periodontitis
BSA	bovine serum albumin
CB	coating buffer
CD	cluster of differentiation
DAB	Diaminobenzidine
EC	endothelial cell
ELAM-1	endothelial cell leukocyte adhesion molecule-1
HBSS	Hank's buffered salt solution
HLA-DR	class II MHC
HUVECs	human umbilical vein endothelial cells
GCF	gingival crevicular fluid
GCW	gingival crevicular washing
GI	gingival index
ICAM-1	intercellular adhesion molecule-1
IEL	intraepithelial lymphocyte
IFN	interferon
IL	interleukin
JE	junctional epithelium
JE/CT	connective tissue subjacent to junctional epithelium
JE/SE	junctional epithelium/sulcular epithelium
JP	juvenile periodontitis
LAD	leucocyte adhesion deficiency

LC	Langerhans cell
LF	lactoferrin
LFA	leucocyte function associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibody
MANOVA	multivariate analysis of variance
MGI	modified gingival index
MHC	major histocompatibility complex
OE	oral epithelium
OE/CT	connective tissue subjacent to oral epithelium
OPD	ortho-phenylene diamine
OTC	organ tissue culture
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-tween 20
PD	probing depth
PI	plaque index
PMN	polymorphonuclear leucocyte
Pre-PP	Pre-pubertal periodontitis
RPP	rapidly progressive periodontitis
SE	sulcular epithelium
TNF	tumour necrosis factor
VCAM-1	vascular cell adhesion molecule-1

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following:

My supervisors, Dr D. F. Kinane and Professor P. C. Wilkinson for their encouragement to undertake this project.

Dr D. F. Kinane for his teaching, guidance, continuous support and supervision throughout the period of research and for performing the analysis of the repeated measures data. For teaching me that computers were not the 'complicated machine, that I could never work' and enlightening me on the finer points of computer literacy. For his persistent belief in my capabilities and for making me realise that I could accomplish almost anything. Finally, I would like to thank him for his critical appraisal of my written work and my thesis in its many drafts.

Professor P.C. Wilkinson for his advice, guidance and continuous support over the period of study, and I would also like to thank him for his critical review of the thesis.

The University of Glasgow Postgraduate Scholarship Fund for providing financial support throughout these studies.

Professor K. W. Stephen, for his support and encouragement and for allowing me to use the facilities of the Periodontal Unit of the Department of Adult Dental Care in collaboration with Dr E. Adonogianaki.

Dr E. Adonogianaki for her support, encouragement and collaboration in many studies, which included performing the clinical parts within the 21-day and 10-day experimental gingivitis studies and the lactoferrin ELISA's in the gingival crevicular washing study. For supplying the description of the lactoferrin sandwich ELISA, Tables 2.3 & 2.4 and Figures 2.5 & 2.6, and for enlightening me on the finer points of basic dentistry. For her constant helpful advice throughout the whole period of study, especially during the writing-up period of this thesis.

Dr D. O. Haskard for providing the adhesion monoclonal antibodies directed against ICAM-1 (6.5B5), ELAM-1 (1.2B6), VCAM-1 (1.4C3) and CD11a (TS 1/22), without which the major part of this work would not have been possible.

Dr M. H. Thornhill for providing the adhesion monoclonal antibodies in collaboration with Dr D. O. Haskard, for his critical appraisal of the adhesion molecule paper, for allowing me to discuss my results with him at length and for his reassurances that everybody working with immunohistochemistry shared the same problems.

Miss M.C. Murray for performing the clinical part of the 10-day experimental gingivitis study.

Mr A. Manos and Mr A. Al-Haerian for providing me with gingivectomy tissue for the organ culture studies.

Mr W. Marshall, Senior Chief MLSO, for making all my big problems into small ones, for his help with the colour photography of the immunoperoxidase sections and for his expert technical advice throughout my period of study.

Professor D. G. MacDonald for his expert help with the photography of the sections used in the adhesion molecule paper and for allowing me to use his computerised imagery planimetry system for enumeration of my sections.

Mrs J. Hope for providing me with never ending support in the early days when I was learning all about immunohistochemical staining.

Finally, I would like to thank all the postgraduate students on the balcony for their helpful comments, advice and friendship throughout the period of study.

DECLARATION

This thesis is the original work of the author.

Naureen Arshad Moughal

SUMMARY

The development of gingival inflammation is not well understood, despite many years of investigation. Accordingly, the main aims of this thesis were, to study the immune and inflammatory cellular infiltrate during the development of gingival inflammation and to examine the effect of proinflammatory cytokines on adhesion molecule expression using gingival organ culture.

Two studies were performed which utilised the experimental gingivitis model. In the model, experimental gingival inflammation was induced in two groups of healthy volunteers by withdrawing oral hygiene procedures and allowing plaque to accumulate undisturbed for 21-days and 10-days. Tissue for analysis was obtained from the experimental gingivitis studies and from patients undergoing routine periodontal treatment. Immunohistology and computerised image planimetry were employed for assessing changes within periodontal cellular infiltrate and adhesion molecules; and a bioassay was used to assess changes in the levels of interleukin-1. Initial studies showed that a single staining avidin-biotin-complex method utilising peroxidase as the substrate, gave the optimal immunohistological staining procedure.

Clinical parameters during the experimental gingivitis studies changed as expected, with the 10-day study demonstrating minimal changes and the 21-day study showing large increases as expected. The 10-day study was aimed at analysing changes during very early 'histological'

inflammation and the 21-day study during 'clinically detectable' inflammation.

The cytokine, interleukin-1 was demonstrated in crevicular fluid during the 21-day study in healthy human volunteers. A sensitive and specific bioassay allowed detection of biologically active interleukin-1 in inflamed gingiva. Interleukin-1 levels increased rapidly with plaque accumulation and in advance of the subsequent gingival inflammation, peaking within 7 days of the start of gingivitis.

Monoclonal antibodies to CD1a (specific for Langerhans cells and thymocytes) and HLA-DR (class II major histocompatibility antigens - (MHC)) were used to identify Langerhans cells within gingival biopsies taken every 7 days of the 21-day study and every 3 days of the 10-day study. HLA-DR antibody stained dendritic cells within the oral epithelium which were morphologically identical to the $CD1a^+$ Langerhans cells. During the 10-day study the number of $CD1a^+$ Langerhans cells and $HLA\text{-}DR^+$ Langerhans cells did not change with time. However, within the 21-day study, Class II MHC Langerhans cell numbers rose and plateaued between day 7 and 14, then decreased to baseline by day 21. As plaque accumulated and inflammation developed there was an initial increase, followed by a decrease in $CD1a^+$ Langerhans cells. It was hypothesised that this may reflect the antigen presenting and migrating role of Langerhans cells to lymph nodes.

The cellular infiltrate of gingival tissue was also analysed and it was found that all of the cell types studied (neutrophils; T-cells and T-cell subsets i.e. helper, suppressor, naive and memory) were present in varying quantities. Periodontal cellular infiltrate was seen within healthy tissue and within experimentally inflamed gingival tissue; and demonstrated preferential accumulation in the connective tissue subjacent to the junctional epithelium. Higher numbers of memory-T cells than naive-T cells were found within healthy gingival tissue; with subsequent inflammation development their was no change in memory-T cells numbers, with a simultaneous decrease in the number of naive-T cells. This suggests that the naive-T cells were maturing into memory-T cells, and in turn are maintaining the numbers of memory-T cells within the gingival tissue.

Vascular endothelium expressed ELAM-1 (endothelial cell leukocyte adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1) both in clinically 'healthy' tissue (day 0) and in experimentally inflamed tissue (day 3 to 10 and day 7 to 21). Positive vessels were found mainly in the connective tissue subjacent to the junctional epithelium where the highest numbers of T cells ($CD11a^+$) and neutrophils were also seen. Although T cells were found in all tissue areas studied, neutrophils were largely concentrated in the junctional epithelium and the subjacent connective tissue but were absent from the oral epithelial region. As the experimental gingivitis lesion developed the number of T cells or neutrophils in the different tissue regions did

not change significantly. The most intense vascular ELAM-1 staining redistributed to the connective tissue subjacent to the junctional epithelium, ICAM-1 demonstrated no bias in its distribution and VCAM-1 showed no difference in staining intensity between junctional epithelium and oral epithelium connective tissue areas. A prominent feature was the intense ICAM-1 positive staining of the junctional epithelium and its absence in the closely adjacent oral epithelium, in both clinically 'healthy' and inflamed tissue. Similar patterns of adhesion molecule expression were seen within diseased periodontal tissue, with adhesion molecules located within the most infiltrated areas.

Gingival vascular adhesion molecules did not exhibit the typical modulation seen in other tissue types, they were present throughout the 10 and 21 days of experimental gingivitis. This suggests one of two possibilities, either the blood vessels in gingiva are functionally specialised and constitutively express ELAM-1 and VCAM-1, plus high levels of ICAM-1 to facilitate leucocyte traffic into the gingival crevice; or that the gingival vessels are permanently activated even in 'health' due perhaps to the constant ingress of bacterial products such as lipopolysaccharide across the junctional epithelium which is a loosely packed highly permeable epithelial barrier.

As changes in interleukin-1 were detected before recognisable gingival changes, interleukin-1 may have potential as an early marker of gingival inflammatory changes. Also, the migration of Langerhans cells within the gingival epithelium may represent an important early

event in the gingival immune response to plaque. Generally, greater numbers of cells were seen in the same areas as the adhesion molecules. The gradient of ICAM-1 in junctional epithelium, with the strongest staining on the crevicular aspect plus the vascular expression of ELAM-1, VCAM-1 and ICAM-1 in both clinically 'healthy' and inflamed tissue may be crucial processes which direct leucocyte migration towards the gingival crevice.

The importance of these adhesion mechanisms in protecting against periodontal disease is highlighted by the rapid and severe periodontitis that characterises leucocyte adhesion deficiency (LAD). The studies presented in this thesis have highlighted the importance of understanding the initiation of gingival inflammation and the kinetics of adhesion molecule expression and their control of leucocyte trafficking. Further studies are required to ascertain if defects in adhesion molecule expression may play a part in determining an individuals susceptibility to periodontal disease.

Chapter 1

Introduction

1.1 Introduction

Periodontal disease, a general term for destructive inflammatory reactions affecting the supporting tissues of the teeth, is one of the most prevalent examples of a chronic inflammatory process afflicting man. The primary aetiological agent in periodontal disease is now known to be the accumulation of bacterial plaque in the periodontal crevice and at the gingival margin (Listgarten, 1988). However, the nature of the organisms present and the virulent factors they produce are likely to be more important than the actual quantity of plaque that has collected (Maiden *et al.*, 1990). It is probably the action of these bacterial products on the underlying connective tissues that elicits the inflammatory response within periodontal disease.

The term periodontal disease, is used to denote any disease involving the periodontium, this includes osseous dysplasias, dermatoses and other generalized disorders producing changes in the epithelial and connective tissues. However, for the purposes of this thesis the term periodontal disease will be confined to gingivitis and chronic periodontitis.

1.2 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.

1.2.1 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 can be divided anatomically into marginal and attached gingival areas (Fig. 1.1). In about 50% of cases the marginal gingiva is demarcated from the adjacent attached gingiva by a shallow linear depression, the free gingival groove (Ainamo & Löe, 1966). In fully erupted teeth the gingival margin (groove) is located on the enamel approximately 0.5 to 2 mm coronal to the cervix (cemento-enamel junction).

In human teeth the gingival margin seldom forms a knife-edged termination against the tooth, but is rounded, this is the orifice of the gingival sulcus. The gingival sulcus is a shallow crevice around the tooth bound by the surface of the tooth on 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 nonkeratinised extension of the oral epithelium, and the bottom of the sulcus is formed by the coronal surface of the junctional epithelium. The junctional epithelium unites the gingival connective tissue

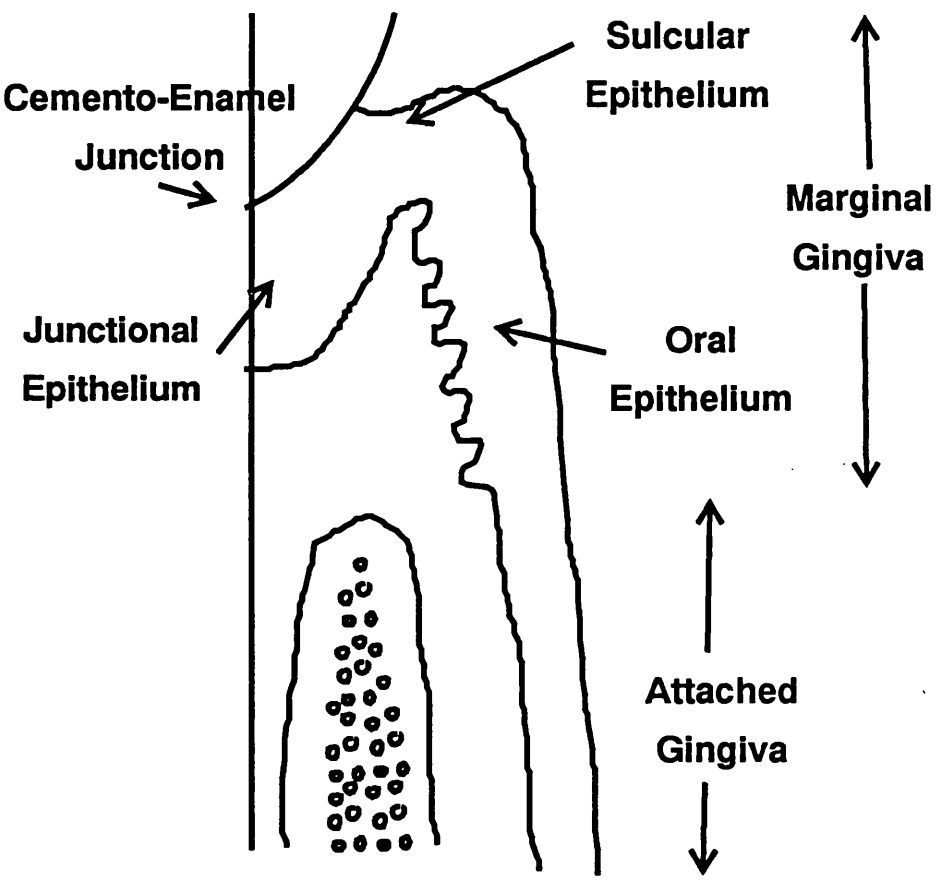

Figure 1.1 Anatomic relationships of normal gingiva.

both with the enamel surface from the cervix of the tooth and with the bottom of the gingival sulcus. The length of the junctional epithelium rarely exceeds 2 to 3 mm.

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) (Fig. 1.2).

1.2.2 Gingival epithelium

The marginal gingiva comprises three areas of epithelium, oral epithelium, the sulcular epithelium and the junctional epithelium (Fig. 1.3). The principal cell type of the oral and gingival epithelium is the keratinocyte. Other cell types found in the epithelium are clear cells or non-keratinocytes, which include Langerhans cells (LCs) (Difranco *et al.*, 1985), Merkel cells (Ness, Morton & Dale, 1987) and melanocytes (Squier & Waterhouse, 1967; Schroeder, 1969).

The keratinocytes constitute about >90% of gingival epithelium and synthesis keratin. The process of keratinisation involves a sequence of biochemical and morphologic events that occur in the cell as it migrates from the basal layer towards the surface (Schroeder, 1981).

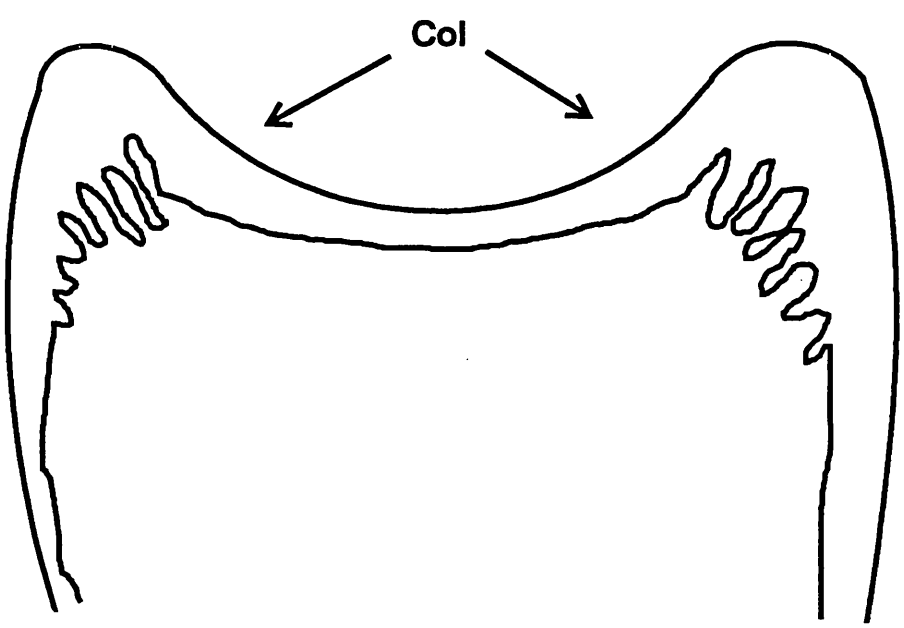

Figure 1.2 The interdental col.

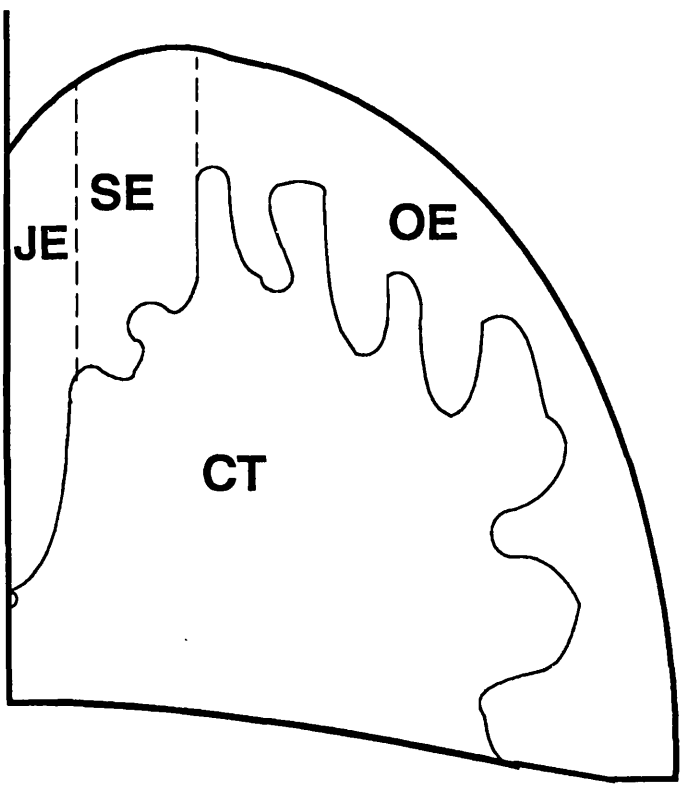

Figure 1.3 Gingival epithelium divisions.

Three types of surface differentiation occurs within the gingival epithelium;

(1) keratinisation i.e. the surface cells form scales of keratin and lose their nuclei,

(2) parakeratinisation i.e. the cells of the superficial layers retain their nuclei and the granular layer is absent,

(3) nonkeratinisation i.e. the cells of the surface layers are nucleated and no signs of keratinisation are present.

1.2.2.1 The oral epithelium

The oral epithelium of the gingiva is keratinised and covers the crest and outer surface of the marginal gingiva, the surface of the attached gingiva and is joined to the underlying connective tissue by a basal lamina (Stern, 1965). The border between the oral epithelium and the underlying lamina propria of the connective tissue is uneven and characterised by deep epithelial ridges that surround finger-like connective tissue papillae (rete pegs) (Fig. 1.3). These ridges and papillae as they appear in histologic preparations represent interdigitating pegs or folds that tend to run horizontally and parallel to the surface of the gingiva (Löe & Karring, 1971).

The oral epithelium of the gingiva is, like epidermis, subdivided into several layer of cells. In the basal layer (*stratum basale, stratum germinativum*) all cells are adjacent to the connective tissue, from which they are

separated by basement membrane (*basal lamina*). The cells are small and more or less cuboidal or columnar in shape. The next several layers of cells constitute the prickle cell layer (*stratum spinosum*) composed of relatively large polyhedral shaped cells. Superficial to the stratum spinosum are several layers of flattened cells that form the granular layer (*stratum granulosum*) and the cytoplasm of these cells characteristically display keratohyalin granules. The most superficial layer is the cornified layer (*stratum corneum*), which consists of flattened cells that have lost their nuclei and most other organelles as they become keratinised (Schroeder & Theilade, 1966) (Fig. 1.4).

1.2.2.2 The sulcular epithelium

The sulcular epithelium lines the gingival sulcus. It is a thin, nonkeratinised stratified squamous epithelium without rete pegs and extends from the coronal limit of the junctional epithelium to the crest of the gingival margin (Fig. 1.3). The sulcular epithelium has the potential to keratinise if (1) it is reflected and exposed to the oral cavity (Bral & Stahl, 1977; Caffesse, Karring & Nasjleti, 1977) or (2) the bacterial flora of the sulcus is totally eliminated (Caffesse, Kornman & Nasjleti, 1980), suggesting that local irritation of the sulcus prevents sulcular keratinisation.

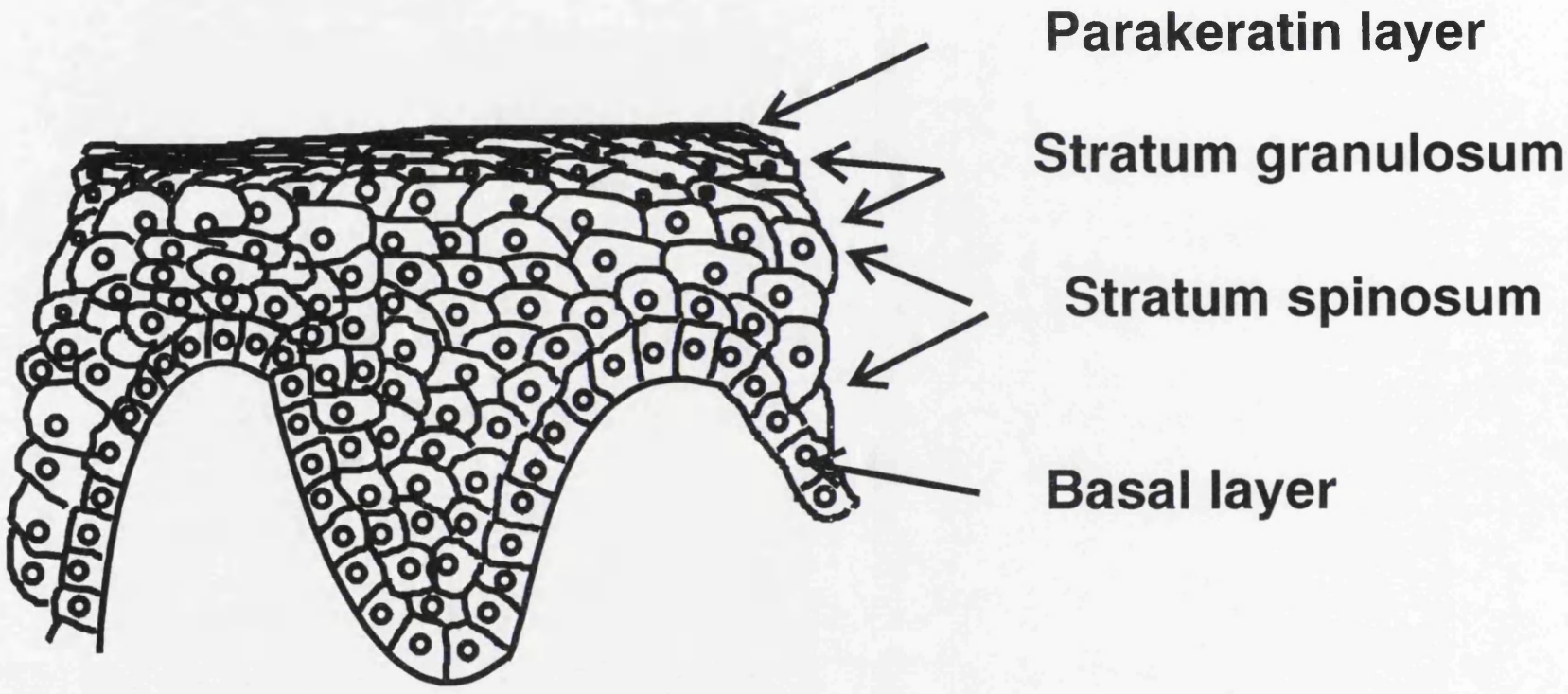

Figure 1.4 Cell layers of stratified squamous epithelium.

1.2.2.3 The junctional epithelium

The junctional epithelium consists of a collar-like band of stratified squamous nonkeratinising epithelium (Fig. 1.3). It is three to four cell layers thick in early life, but the number of layers increases with age to 10 or even 20; its length ranges from 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) that is comparable with that which attaches epithelium to 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. Polymorphonuclear leucocytes (PMNs or neutrophils) are found routinely in the junctional epithelium of both conventional and germ-free rats (Yamasaki *et al.*, 1979).

1.2.3 The gingival connective tissue

The connective tissue (lamina propria) of the gingiva consists primarily of cells, fibres, nerve processes and blood vessels embedded in dense collagenous connective tissue. The connective tissue of the gingiva is densely collagenous, containing a prominent system of collagen fibre bundles called the gingival fibres which are arranged in three groups; gingivodental, circular and transseptal (Arnim & Hagerman, 1953). The main cell is the fibroblast, which synthesises the basic elements of the connective tissue. Other cells found include undifferentiated

mesenchymal cells, mast cells (Shelton & Hall, 1968) and macrophages.

Leucocytes have been found in 'clinically' healthy gingival sulci in humans and experimental animals. The predominant leucocyte found is the PMN, they appear in small numbers extravascularly in the connective tissue adjacent to the bottom of the sulcus; from there they travel across the epithelium (Cattoni, 1951; Grant & Orban, 1960; Thurre *et al.*, 1984) to the gingival sulcus where they accumulate and are eventually lost to the oral cavity (Fig. 1.5). Leucocytes are present in the sulcus, even when histological sections of adjacent tissue are free of inflammatory infiltrate (Attström, 1970).

1.2.4 Vessels within the gingiva

The vessels in the gingival connective tissue are of two main types, blood or lymphatic vessels.

1.2.4.1 The blood supply of the gingiva

The blood vessels in the gingival connective tissue are arterioles, capillaries, and small veins. Capillaries terminating immediately below the basement membrane provide the nutritional supply for the gingival epithelium. Microscopic studies of the gingival surface *in vivo* have shown that there are approximately 50 capillaries per square millimetre, each of which terminates in a loop in the peripheral part of the connective tissue papillae

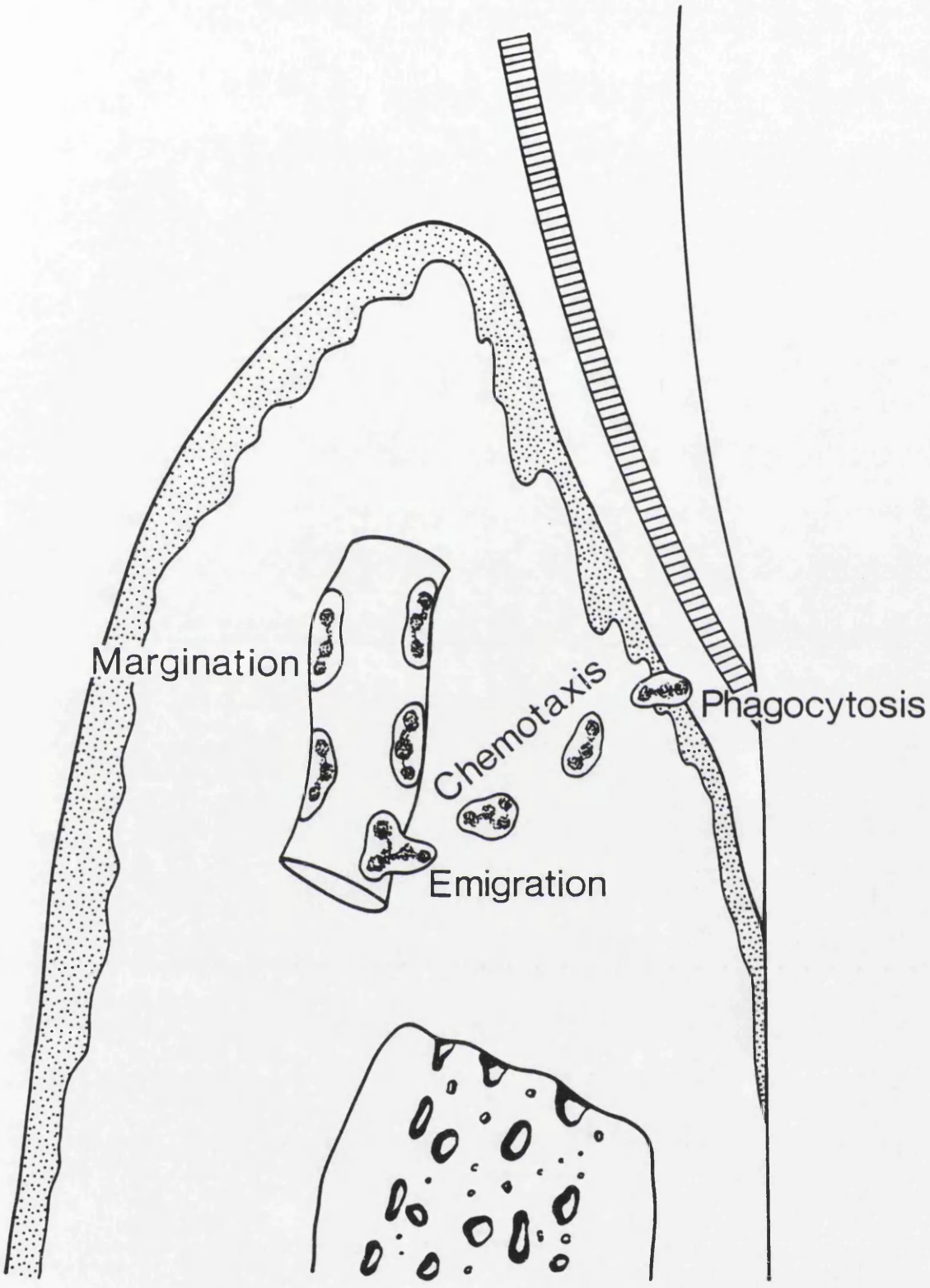

Figure 1.5 Neutrophils traversing from the blood vessels through the connective tissue to the gingival crevice.

adjacent to the epithelial border. Wide variation in numbers exist as well as between individuals. However, longitudinal studies have shown that within a particular gingival region the same vascular pattern persists over a long period of time. Indicating that under normal circumstances the blood supply is quite consistent with respect to the number, distribution, and size of the blood vessels (Karring & Löe, 1967).

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). Next to the junctional epithelium and sulcular epithelium the terminal blood vessels form a plexus that extends under the epithelial surface from the gingival margin to the apical extension of the junctional epithelium (Egelberg, 1966).

1.2.4.2 The lymphatic drainage of the gingiva

The lymphatic drainage of the gingiva involves the lymphatics of the connective tissue papillae. The lymph progresses into the collecting network external to the periosteum of the alveolar processes and then to the regional lymph nodes (particularly the submaxillary group). In addition, lymphatics just beneath the junctional epithelium extend into the periodontal ligament and accompany the blood vessels.

1.2.5 Sulcular fluid

In 1960, Brill & Brönnestam carried out a series of experiments. These studies initiated interest in studying the composition of sulcular fluid and they showed that after performing immunoelectrophoretic analyses that at least seven different plasma proteins could be shown to be present within the fluid. These results indicated that small molecules were passing from the subepithelial connective tissues into the gingival crevice and out into the oral cavity. Also, Brill, (1959) confirmed the presence of sulcular fluid (gingival crevicular fluid - GCF) in humans and considered it a transudate. Later, other workers confirmed that the sulcular fluid was an inflammatory exudate and not a continuous transudate (Löe & Holm-Pedersen, 1965; Weinstein *et al.*, 1967).

Fluid and plasma proteins leak from the microvasculature percolating through the connective tissue and junctional epithelium into the gingival sulcus to give rise to the gingival fluid (Egelberg, 1967); and in crevices which have no gingival inflammation, there is no exudation or fluid according to Oliver, Holm-Pedersen & Löe, (1969).

The flow of GCF has been shown to have a close relationship with capillary permeability, with fluid passing from the subepithelial connective tissues between or through the cells of the junctional epithelium into the crevice (Brill & Brönnestam, 1960). With Schroeder, Rossinsky & Listgarten, (1989) confirming recently that the junctional

epithelium of human gingiva is the pathway for inflammatory exudation.

Analysis of constituents of GCF provides information about the nature of the host response in the periodontium (Offenbacher *et al.*, 1984; Smith *et al.*, 1985) and offers to provide a means of monitoring the progression of chronic inflammatory periodontal disease (Fine & Mandel, 1986). Two techniques are most commonly employed for collection of GCF; they are (a) pre-cut methyl cellulose filter paper strips and (b) microcapillary tubes (Cimasoni, 1983). Collection of GCF is frequently accomplished with the intra-crevicular placement of strips (Cimasoni, 1983). Problems with sampling GCF can be encountered as repeated sampling of the same site results in changes in GCF flow rate (Mann, 1963; Cimasoni, 1983) and also that repeated placement of filter strips results in changes in the subjacent microvasculature (Egelberg, 1966).

1.2.6 Clinical indices

Clinical indices have been used to study the extent and severity of gingivitis for many years. Two types of Index systems are commonly used when clinical assessment of gingiva is required, one measures the presence of plaque and the other gingival involvement. Pocket depth assessment on the other hand, is used more in assessing periodontal destruction and is an indicator of disease experience rather than the current situation.

1.2.6.1 Plaque index

Plaque Indexes (PI) generally estimate the soft accumulations on teeth either in terms of tooth area covered or in thickness of the material in the area measured (reviewed by Fischman, 1986). The indices are generally quantified non-linearly and the scores assigned on an arbitrary scale. This plaque scoring system can be used on a whole mouth or on a selected tooth or site basis.

1.2.6.2 Gingival index

Gingival indexes (GI) measuring gingival involvement have relied on one or more of the following criteria; gingival colour, bleeding, gingival crevicular fluid flow, contour, bleeding-upon-probing *etc.* Indices using bleeding-upon-probing (Löe & Silness, 1963) are widely used, with bleeding being used as an indicator of the extent of gingival inflammation. Indices using bleeding-upon-probing are invasive and prevent histological assessment of untraumatised gingival tissue. The modified gingival index (MGI) of Lobene *et al.* (1986) is a modification of this GI (Löe & Silness, 1963). It omits the bleeding-upon-probing component of the index and makes it more sensitive to early changes in gingiva, increasing the sensitivity of the lower scores. The MGI does not disturb plaque accumulation, causes minimal irritation and injury to the soft tissues and is therefore ideally suited for use in longitudinal studies (i.e. experimental gingivitis studies)

where gingival biopsies are taken for histological assessment.

1.2.6.3 Pocket depth

Probing pocket depth, recession and loss of attachment are parameters used to evaluate the amount of tissue lost in periodontal disease and permit quantification of the apical extension of the inflammatory lesion.

1.3 Periodontal health and disease

1.3.1 Introduction

Prior to the decade of the 1960's, very little research was performed on gingivitis. However following the development of the Periodontal Index of Russell, (1959), the Periodontal Disease Index of Ramfjord & Arbor, (1959), and other similar indices, many epidemiologic studies assessing the prevalence and severity of gingivitis and periodontitis have been carried out. Many features of periodontitis have been elucidated but some mechanisms still remain unresolved. The following sections will outline what is known about periodontitis and will also highlight the deficient areas which require further research.

1.3.2 Bacterial plaque

It has now generally been accepted that substances in bacterial plaque are the prime aetiological agents responsible for gingivitis and periodontitis (Listgarten, 1988; Christersson, Zambon & Genco, 1991); and theories

exist which propose that different forms of, and the severity of, periodontal disease can be related to shifts in the proportions of bacterial species within plaque (Newman, 1990). A classical demonstration of the importance of microbial plaque in gingivitis was made by Löe, Theilade & Jensen, (1965) using their experimental gingivitis model. They demonstrated that gingival inflammation in healthy gingiva could be induced by the accumulation of microbial plaque and that removal of the plaque resulted in resolution of the lesion. With the microbiological aetiology of the disease now confirmed in studies using human and experimental animal models (Payne *et al.*, 1975; Lindhe & Rylander, 1975; Page & Schroeder, 1976; Moore *et al.*, 1982, 1984; Ohta *et al.*, 1991), plaque has been accepted as the causative agent in gingival inflammation.

Even though it has been demonstrated that good oral hygiene can prevent the development of gingival inflammation and periodontal disease (Saxe *et al.*, 1967; Suomi *et al.*, 1969, 1971a; Suomi, Leatherwood & Chang, 1973), not all the characteristics of the disease can be accounted for by dental plaque. For example periodontal lesions can occur in the absence of plaque (Van der Waal, 1991), variation in susceptibility between animal species and between humans can not be explained by plaque alone (Fish, 1935; Page, Simpson & Ammons, 1975; Ammons, Schectman & Page, 1972) and prevalence from tooth to tooth (site specificity) as

exemplified by the distributions of lesions in juvenile periodontitis is not explained. This suggests that host defense mechanisms may play an important role in the pathogenesis of periodontal disease.

1.3.3 Defence mechanisms operating in the gingiva

Various defence mechanisms are in operation around the gingival sulcus, and elucidation of their role has been greatly helped by studies on germ-free rats and dogs and on animals whose teeth have been kept meticulously clean (Attström & Egelberg, 1970, 1971; Attström, 1971; Listgarten & Heneghan, 1971, 1973; Attström, Graf-De Beer & Schroeder, 1975; Schroeder, Graf-De Beer & Attström, 1975; Garant, 1976a, 1976b).

The intact epithelial barrier is one of the host defence mechanisms which prevents bacteria entering the connective tissue, however large molecules can enter via the junctional epithelium (Steinberg, Joseph & Evans, 1981). Any noxious substances which manage to penetrate the epithelial barrier are dealt with by phagocytosis and other defense mechanisms operative within the connective tissue.

One of the earliest events in gingivitis is 'ulceration' (Schroeder & Attström, 1979) of the wall of the gingival sulcus resulting in an influx of microbial substances into the underlying connective tissue. Saliva, another of the host defence mechanisms, contains secretory IgA antibody,

peripheral blood leucocytes capable of phagocytosis and killing; agglutinins which can cause clumping and clearance via nonspecific interactions and its flushing action helps clear bacteria from the oral cavity. Once inflammation begins, gingival fluid exudes from the gingival sulcus (Löe & Holm Pedersen, 1965), this can be protective as it has uni-directional flow resulting in a flushing action and contains all the substances present in blood serum but in lower concentrations. Complement proteins are present within the gingival exudate and complement is activated during gingival inflammation (Schenkein & Genco, 1977a, 1977b; Okada & Silverman, 1979). Activation of complement leads to enhancement of phagocytosis and killing of bacteria as well as initiation and perpetuation of the inflammatory response. Complement components can play a role in tissue damage such as bone resorption (Raisz *et al.*, 1974), as well as in healing and connective tissue regeneration (Bordin, Page & Narayana, 1984).

GCF contains nonspecific opsonins and antibody specific for determinants of pocket bacteria, these may participate in host defence by enhancing microbial killing and clearance. Antibody specific for periodontal pocket bacteria comes from two sources, peripheral blood or it is locally produced by plasma cells present within the soft tissues of the pocket wall (Lally, Baehni & McArthur, 1980; Mouton *et al.*, 1981; Schonfeld & Kagan, 1982; Daly, Cripps & Clancy, 1983b). Gingival plasma cells produce two types of

antibodies, antibodies that react specifically with periodontal pocket bacteria, as well as non-specific antibody resulting from polyclonal activation (Page & Schroeder, 1981).

Another host mechanism which can be protective is the high turnover rate of sulcus tissue, junctional epithelium, matrix components of the gingival connective tissue and periodontal ligament. These tissue components have a high regeneration potential and thus the periodontium can accommodate considerable amounts of damage without long term deleterious consequences.

1.3.4 Neutrophil traffic into the gingival crevice

Neutrophils are important in the prevention of the development of gingivitis and formation of gingival and periodontal pockets. It has been suggested that in normal conditions a constant stream of neutrophils (PMNs) migrate from the vessels of the gingival plexus through the junctional epithelium to the gingival margin into the gingival sulcus and oral cavity (Ryder, 1980a, 1980b, 1980c). Most bacteria produce substances that chemotactically attract neutrophils (Lareau, Herzberg & Nelson, 1984) and chemotactic substances are also present within the saliva. A chemical gradient of chemotactic agents seems to exist across normal, intact junctional epithelium and connective tissue. Neutrophils on leaving the blood vessels are guided by this gradient towards the

gingival margin or into the gingival sulcus where they become functional (Kowolik & Raeburn, 1980; Kowashi, Jaccard & Cimasoni, 1980; Charon *et al.*, 1982b; Thurre *et al.*, 1984) (Fig. 1.5). These neutrophils, together with the epithelial barrier could be considered as the first line of defence at the gingival tooth junction.

Local and systemic host defence mechanisms can usually control microbial challenge around the teeth and prevent breakdown of the periodontal apparatus. Tissue response may be seen as gingival inflammation and the damage caused is minimal, which is usually repaired by the high turnover rate of the tissue components. No pockets form and periodontal disease does not occur. It is easy to understand how gingivitis and periodontal disease can occur in an individual who has an underlying or pre-existing defect in their primary front line defence system (ie neutrophil dysfunction) (Genco *et al.*, 1986) but it is more difficult to explain why gingivitis/periodontitis occurs in apparently healthy individuals.

1.3.5 Clinical, morphologic and functional changes within gingiva as inflammation develops

Inflammatory changes in the gingiva develop within a couple of days of undisturbed bacterial growth on the cervical portion of the tooth surface. Clinical inflammation is initially seen as discrete colour and texture changes of marginal tissues. After ten to twenty days of plaque

accumulation overt gingivitis is established in most individuals and is characterised by gingival redness and swelling. The soft tissue have an increased tendency to bleed on gentle probing.

Morphologic and functional changes in the gingiva during plaque accumulation have been thoroughly investigated using both humans and experimental animal models, especially beagle dogs (Zachrisson, 1968; Schroeder, 1970a, 1970b; Attström & Egelberg, 1971; Lindhe, Hamp & Löe, 1973; Schroeder, Münzel-Pedrazzoli & Page, 1973; Lindhe & Rylander, 1975; Payne *et al.*, 1975; Schroeder *et al.*, 1975; Schroeder & Lindhe, 1975; Schroeder & Attström, 1979). Based on qualitative as well as morphometric point-counting procedures used for stereologic analysis of the gingival and periodontal tissue (Schroeder & Graf-De Beer, 1976), and combined with previous observations (Schroeder, 1970b; Page & Schroeder, 1973; Lindhe *et al.*, 1974; Page *et al.*, 1974; Attström *et al.*, 1975; Page *et al.*, 1975; Payne *et al.*, 1975; Schroeder *et al.*, 1975), 5 different states ranging from health to advanced disease of the periodontium have been proposed. This classification was initially proposed by Page & Schroeder, (1976), the stages were divided into health, the initial lesion, the early lesion, the established and the advanced lesion. Page & Schroeder, (1976) proposed that the 'initial' infiltrate of PMNs was rapidly replaced within 48 hours by a lymphocytic lesion, which persisted, unless further challenged by exogenous

stimuli from plaque. With further plaque challenge, the composition of the cellular infiltrate, was then suggested to transform into a plasma cell dominated lesion. At which stage tissue destruction including bone resorption was suggested to occur. Recent ideas on periodontal disease activity (discussed in section 1.5) and the fact that not all postulated stages have been observed histologically (Zachrisson, 1968, Payne *et al.*, 1975, Seymour, Powell & Aitken, 1983a; Seymour *et al.*, 1983b; Brecx *et al.*, 1987b); and the inability to test the models validity with true longitudinal studies, brings into question Page & Schroeder's, (1976) model.

The following sections will review the current thoughts on the histopathology of each stage from health to disease, making references to Page & Schroeder, (1976) model where appropriate.

1.3.5.1 Healthy gingiva

Normal gingiva is characterised clinically by its pink colour, firm consistency, scalloped outline of the gingival margin and firm interdental papillae (Orban, 1948; Ainamo & Löe, 1966), which does not bleed on gentle probing and occupies the entire space available below the contact area of the neighbouring tooth.

Normal gingiva is relatively free from inflammatory cell accumulation and the gingival sulcus is rarely evident at

the microscopic level in these tissues. The oral surface is covered with keratinised oral epithelium which fuses with the junctional epithelium. The latter is firmly attached to the tooth surface via hemidesmosomes and the marginal portion of the junctional epithelium harbours few neutrophilic granulocytes and mononuclear cells. The space between the oral epithelium and junctional epithelium is normally occupied by collagenous structures, immediately below the junctional epithelium there is a microvascular plexus, containing numerous venules (Egelberg, 1967). Isolated inflammatory cells may be present in this area and/or around some vessels in more distant locations (Attström *et al.*, 1975; Lindhe & Rylander, 1975).

About 40% of the normal gingiva volume is occupied by epithelial structures (oral epithelium - 30%; junctional epithelium - 10%) and the remaining 60% volume is occupied by connective tissue components such as collagen fibres, matrix cells, vessels and nerves.

1.3.5.2 Clinically 'healthy' gingiva

Under experimental conditions clinically 'healthy' gingiva can be established. The tissues are characterised by GI scores of 0 and only a trace of gingival exudate (Löe & Holm-Pedersen, 1965; Oliver *et al.*, 1969), but this does not correspond with histologically 'normal' gingiva (Brecx *et al.*, 1987a). Histologically normal gingival tissue is only found adjacent to relatively plaque-free teeth and

therefore is relatively rare in humans, but can be found in germ-free animals (Listgarten & Heneghan, 1971); and also in animals whose gingiva have been kept meticulously clean (Lindhe & Rylander, 1975). Biopsies of clinically normal human gingiva exhibit inflammatory cell infiltration. These leucocytes appear to be important in the day-to-day host response to bacteria and other substances to which the gingiva are exposed. This small but definite infiltrate of inflammatory cells can be detected in the coronal portion of the connective tissue, and as well as containing lymphocytes, predominantly T-cells with very few B-cells or plasma cells (Page & Schroeder, 1976; Seymour *et al.*, 1983a, 1983b), it also contains PMNs, monocytes and macrophages. It occupies around 3-5% of the connective tissue volume and is in contact with the junctional epithelium. Collagen is depleted in this infiltrated area and the tissue compartment is rich in vascular structures. Fluid and plasma proteins leak from the microvasculature percolating through the connective tissue and junctional epithelium into the gingival sulcus to give rise to the gingival fluid (Egelberg, 1967). In the vast majority of crevices which have no gingival inflammation, no exudation or fluid is seen. This phenomena was demonstrated by Oliver *et al.* (1969) when they grouped gingival areas according to their gingival index score (Löe & Silness, 1963). Using filter paper strips to collect the GCF, it was shown that 65% of sites with a GI=0 demonstrated no gingival exudation, with the remaining sites (35%)

demonstrating only traces amounts of exudation; whereas all sites with a GI>1 demonstrated gingival exudation. They concluded that GCF could be collected from all sites with clinically detectable gingival inflammation (i.e. GI scores >1), and sites with no gingival inflammation (i.e. GI=0) exhibited no exudation of GCF; but when present, it could only be collected in trace amounts.

One of the major problems in determining the pathogenesis of periodontal disease is the inability of investigators to distinguish clearly between normal and pathologically altered tissue. At what point disease actually commences has not been determined and in the absence of any definitive evidence two possibilities exist. Firstly, features seen in the initial lesion are merely reflecting enhanced levels of activity of host defence mechanisms normally seen within the gingival tissues (Attström *et al.*, 1975; Payne *et al.*, 1975; Schroeder *et al.*, 1975; Page & Schroeder, 1976), or that the 'initial' stage is reflecting a quiescent phase which follows the destructive disease; and thus may be a later or a constantly recurring stage in the progression of the disease (Gillett *et al.*, 1990).

1.3.5.3 The initial lesion - gingivitis

Following plaque accumulation one of the earliest reactions seen is characteristically an acute exudative inflammatory response known as the *initial lesion* (Payne *et al.*, 1975; Schroeder *et al.*, 1975) as classified by Page & Schroeder,

(1976). The initial lesion is localised around the gingival sulcus and usually emerges after 2-4 days of plaque accumulation, but the changes can be seen within 24 hours of plaque accumulation and these usually consist of vascular changes beneath the junctional epithelium. More blood is brought to the area due to dilation of the arterioles, capillaries and venules of the dentogingival plexus. Simultaneously, intercellular gaps between capillary or venular endothelial cells appear due to elevation of the hydrostatic pressure, resulting in increased permeability of the microvascular bed to fluids and proteins which leak into the tissues. This results in an increase in gingival crevicular fluid flow seen both in animal models and human models (Payne *et al.*, 1975; Schroeder *et al.*, 1975). This increase in fluid flow may function as a defence mechanism, removing bacterial products from the gingival tissues by diluting and flushing them out and by supplying antibacterial serum components such as antibodies and complement to the area. Usually non-inflamed gingiva has minute concentrations of gingival crevicular fluid, so the amount of fluid present and the concentration of specific plasma proteins in the gingival sulcus could be used to estimate the extent of inflammation (Fine & Mandel, 1986). As the vascular changes occur numerous neutrophils, monocytes/macrophages as well as lymphoid cells begin to migrate from the dentogingival microvascular plexus and the cellular response is well established by 2 to 4 days (Lindhe *et al.*, 1973; Listgarten

& Ellegaard, 1973; Payne *et al.*, 1975; Schroeder *et al.*, 1975; Brecx *et al.*, 1987b), and is reversible if the exogenous stimuli i.e. plaque is removed.

The nature of the initial lesion is relatively well understood. *In vitro* and *in vivo* studies have demonstrated that extracts of microbial plaque from humans, as well as sonic extracts of periodontal bacteria are chemotactic for leucocytes (Hellden & Lindhe, 1973; Wennström *et al.*, 1980; Lareau *et al.*, 1984) and the initial lesion can be induced by their application to the gingival sulcus region in otherwise normal animals. 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.*, 1988a); this is in contrast to the findings with animal models where plasma cells are found to predominate apically to the gingival sulcus (Listgarten & Ellegaard, 1973). It has been suggested that host defense mechanisms may not always have a beneficial role, they may be destructive and although neutrophils and macrophages are recruited to the area to defend the host against bacterial attack, their accumulation in the coronal part of the connective tissue and junctional epithelium probably accounts for much of the damage seen in this portion of the gingiva (Lehner, 1972; Page & Schroeder, 1973; Genco *et al.*, 1974; Horton, Oppenheim & Mergenhagen, 1974). A feasible mechanism of this destruction of collagen in dogs, could be the activity of collagenase and other enzymes

released by the infiltrating and transmigrating neutrophils during the initial stage of gingivitis (Attström & Schroeder, 1979; Schroeder & Attström, 1979). Such a loss of collagen however,is not seen in humans at this early stage (Brecx *et al.*, 1987b). These discrepancies in the development of inflammation at this early stage, questions the role of animal models in studying the development of human gingival and periodontal inflammation.

1.3.5.4 The early lesion - gingivitis

The *early lesion* evolves from the initial lesion within about one week following the beginning of plaque accumulation (Schroeder *et al.*, 1973; Payne *et al.*, 1975). The vessels of the coronal portion of the dentogingival plexus remain dilated (Egelberg, 1967; Lindhe & Rylander, 1975) and with additional plaque accumulation there is more pronounced infiltration of PMNs and monocyte/macrophages within the dentogingival epithelium, compared with the initial lesion. The junctional epithelium now contains increased numbers of transmigrating neutrophilic granulocytes and infiltration of mononuclear cells, especially lymphocytes (Listgarten & Ellegaard, 1973; Schroeder *et al.*, 1973; Payne *et al.*, 1975; Schroeder *et al.*, 1975). The inflammatory cell infiltrate at this stage has increased to occupy approximately 10-15% of the connective tissue volume of the free gingiva (Schroeder *et al.*, 1973) and it contains small and medium sized lymphocytes, many of which are T-cells (responsible for

cell mediated immune reactions), while others represent B cells (which will probably develop into antibody producing plasma cells) (Seymour *et al.*, 1983b). Lymphocytes predominate in the early lesion and only a few plasma cells have been demonstrated (Listagarten & Ellegaard, 1973; Payne *et al.*, 1975; Seymour *et al.*, 1983b; Brecx *et al.*, 1987b). Clinically the early lesion may appear as gingivitis, the gingival fluid flow and the numbers of transmigrating leucocytes reach their maximum between 6 and 12 days after the onset of clinical gingivitis (Lindhe *et al.*, 1973).

Some fibroblasts within the inflammatory cell infiltrate appear to be altered (Page & Schroeder, 1973; Simpson & Avery, 1973, 1974; Page *et al.*, 1975), whilst fibroblasts in adjacent noninflamed connective tissue have been shown to have normal ultrastructural features (Simpson & Avery, 1973, 1974). The fibroblasts become pathologically altered as evidenced by electron-lucent nuclei, swollen mitochondria and vacuolization of the endoplasmic reticulum with rupture of the cell's membrane. The altered cells have been shown to be intimately associated with activated lymphocytes, suggesting a cell mediated killing mechanism (Schroeder & Page, 1972; Simpson & Avery, 1974). Presumably some of these fibroblasts have been injured by cytotoxic products such as lymphotoxin produced primarily by lymphoid cells (Granger & Williams, 1968; Schectman *et al.*, 1972; Walker & Lucas, 1972).

Furthermore the basal cells of the junctional/sulcus epithelium have proliferated and rete pegs have been seen piercing and entering the coronal part of the infiltrate (Schroeder, 1970a; Schroeder *et al.*, 1973; Horton *et al.*, 1974; Simpson & Avery, 1974; Payne *et al.*, 1975; Schroeder *et al.*, 1975; Page & Schroeder, 1976). The character of the cellular infiltrate and the nature of the pathological alterations observed, has lead to the concept that cellular hypersensitivity may be an important component of the early lesion. Wilde, Cooper & Page, (1977) demonstrated that typical early lesions could be created in the gingival tissue of rats and monkeys sensitised to skin contact antigens followed by challenge at the gingival margin with the same antigen. A specific T-cell mediated mechanism was suggested, as sensitization can only be transferred to unsensitised animals by means of lymphocytes, but not by serum from the sensitised animals.

The duration of the early lesion has not been definitively determined, Seymour *et al.* (1983a, 1983b) studied biopsies from individuals undergoing a period of experimental gingivitis for 21 days, they found the initial infiltrate to consist mostly of lymphocytes, approximately 70% of which were T-cells. Although the size of the lymphoid cell infiltrate did increase during the 21 day course of the experiment, the composition did not change. Recently a six-month experimental gingivitis study carried out by Brecx *et al.* (1988a) reported that greater than six months

of oral hygiene abstention is required, if plasma cells are to dominate the lesion. Thus, the early lesion may persist for longer time periods than previously suspected. Though some studies have been carried out in humans, where biopsies have been taken in a cross-sectional manner from children suffering from gingivitis (Schroeder *et al.*, 1973; Longhurst, Gillett & Johnson, 1980), and these studies have suggested that lesions are plasma cells dominated. It must be noted, however, that childhood gingivitis lesions (plasma cell dominated) are different from adult gingivitis lesions, which are lymphocyte dominated (Payne *et al.*, 1975; Brecx *et al.*, 1987b). The biopsies in the studies were taken in a cross-sectional manner and divided into groups according to gingival index scores; it is therefore impossible to determine for what length of time inflammation has been developing at these sites. Since clinical parameters have been shown to reach a maximum after 4 months of oral hygiene abstention (Brecx *et al.*, 1988a), the sites in the aforementioned studies may have been exposed to plaque for an undeterminable period of time, and therefore may not represent an early lesion i.e. as suggested by Page & Schroeder model, (1976).

1.3.5.5 The established lesion

The *established lesion* as defined by Page & Schroeder, (1976) is hypothesised to be predominated by plasma cells. Many investigators have supported this hypothesis by stating that only 3-4 weeks of plaque accumulation is

needed for a plasma cell dominated lesion to be formed (Zachrisson, 1968; Payne *et al.*, 1975). This concept has been challenged by Brecx *et al.* (1988a), after carrying out a six-month experimental gingivitis study, where they concluded that greater than six months of plaque accumulation was needed before the lesion became plasma cell dominated. The exact period of time required for lesions in humans to become plasma cell dominated is still unknown, though 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 evolves from the early lesion, probably in conjunction with the creation of a small gingival pocket lined with pocket epithelium (Schroeder & Lindhe, 1975). This phase of the lesion development is commonly referred to as 'chronic gingivitis'. Continued exposure to plaque results in increased fluid exudation from the vasculature and increased numbers of leucocytes migrating into the tissues and the gingival crevice and persistence of plaque sustains the inflammatory reaction within the coronal gingiva.

In the established lesion, features seen in the earlier stages of the lesion are still present but in an accentuated form (Mulvihill *et al.*, 1967; Simpson & Avery, 1974). Large numbers of mature plasma cells are seen (Freedman, Listgarten & Taichman, 1968; Simpson & Avery,

1974) and these are situated primarily in the coronal connective tissue as well as around vessels in more distant parts of the gingival connective tissue. Collagen loss continues in both apical and lateral directions as the inflammatory cell infiltrate expands, resulting in collagen-depleted areas radiating deeper into tissues (Page & Schroeder, 1973). As gingivitis develops the overall size of the connective tissue infiltrate increases, proliferation of the dentogingival epithelium is maintained and the rete pegs extend into the connective tissue infiltrate. The pocket epithelium is not attached to the tooth surface and forms the external boundary for the inflammatory cell infiltrate. It is also heavily infiltrated with PMNs, macrophages, lymphocytes and plasma cells. Most of the neutrophils, eventually migrate across the epithelium into the gingival pocket. The pocket epithelium may be extremely thin and may actually be ulcerated in some areas. It is also fragile and more permeable to the passage of substances than the junctional epithelium (Thilander, 1964). Removal of plaque at this stage only results in healing of the lesion by repair, and not resolution as seen in the earlier stages (Mackler *et al.*, 1977; Okada, Kida & Yamagami, 1983).

Established lesions of 2 types appear to exist, some remain stable and do not progress for months or years (Lovdal, Arno & Waerhaug, 1958; Suomi, Smith & McClendon, 1971b; Lindhe, Hamp & Löe, 1975; Page *et al.*, 1975), while others

appear to become more active and convert to progressive destructive lesions. The nature of this conversion has been studied, but it is not understood (Schroeder & Lindhe, 1975). Conflicting views exist, Seymour, Powell & Davis, (1979) hypothesised that it is a change from T-cell dominance to B-cell dominance in this infiltrate, which converts such a lesion from a stable state to an active state with aggressive destruction. This view is not shared by Page, (1986) nor does it fit with the results of Gillet, Cruchley & Johnson, (1986) who find mainly B-cell infiltrate associated with the non-progressive lesions in childhood gingivitis. More recently however, Walsh *et al.* (1987a) suggest that lesions of chronic gingivitis in children are T-cell dominated. Further analysis of the techniques used within these studies sheds some light on the conflicting results. Gillet *et al.* (1986) in their study used a monoclonal antibody directed against HLA-DR to separate their lymphoid population into two large groups $HLA\text{-}DR^{+}$ and $HLA\text{-}DR^{-}$ populations. They found that in childhood gingivitis two groups of cells of equal size were obtained, one positive for the marker and one negative for the marker. They proposed that the $HLA\text{-}DR^{+}$ cells were B-lymphocytes and the $HLA\text{-}DR^{-}$ cells were unactivated T-helper cells. From their study they concluded that the lesions of childhood gingivitis were B-cell dominated; but on further analysis it can be demonstrated that many assumptions were made by the investigators. The major flaw in their study was pointed out by the investigators themselves, they

highlight the point that the HLA-DR$^+$ population contains untransformed B cells, as well as activated T-helper cells. Therefore, the T-helper cells can be present in either cell pool depending upon their activation status. Walsh *et al.*'s (1987a) study was better designed, a panel of eleven monoclonal antibodies were used to differentiate the population of cells present in childhood gingivitis lesions. They concluded from their study that the non-progressive lesions were T-cell dominated (Seymour *et al.*, 1982). Supporting the view of Seymour *et al.* (1982) and the hypothesis proposed by the same group in 1979 (Seymour *et al.*, 1979).

1.3.5.6 The advanced lesion - periodontitis

The last stage of the lesion is known as the *advanced lesion*. The histopathological and ultrastructural features of this lesion have been investigated thoroughly (Znamensky, 1902; James & Counsell, 1927; Fish, 1935; Freedman *et al.*, 1968; Page *et al.*, 1975), and it has been shown that with continued plaque growth along the crown and the cementum of the root surface there, is a deepening of the periodontal pocket which is accompanied by the proliferation of the dentogingival epithelium over the 'detached root' surface. The advanced lesion has characteristic features, alveolar bone loss, 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.

Conflicting views existed about the type of cell that predominates in the advanced lesion. Schroeder & Lindhe, (1975) studied in dogs, the conversion of the stable established lesion into the advanced destructive lesion of chronic periodontitis. They observed that the lesion changed from a lymphocyte and plasma cell rich established lesion into an advanced lesion which was histologically a plasma cell dominated lesion. This view of the advanced lesion was questioned by Okada, Kassai & Kida, (1984) they found that the predominant cells in the lesion were T-cells and not plasma cells; but their study on humans suffering from adult periodontitis did not look for the presence of B cells or plasma cells, so the study had an inbuilt bias. It has now been 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). The first indication of alveolar bone destruction can be histologically seen in the advanced lesion and is evidenced by bone loss and fibre attachment loss. Development of periodontitis can be initiated using experimental systems but only in experimental animal models (Lindhe *et al.*, 1973), not in human models (Brecx *et al.*, 1988a), thus further questioning the validity of using

animal models to study the development of gingivitis and periodontitis, and extrapolating to the human situation.

1.4 Classification of Periodontal diseases

Periodontal disease is broadly classified into two distinct entities depending upon the extent of the lesion. In gingivitis the inflammatory lesion is confined to the tissues of the marginal gingiva, and in periodontitis the deeper tissues (alveolar bone, periodontal ligament and cementum) are involved (Lyons, Bernier & Goldman, 1959, Socransky *et al.*, 1984; Kornman, 1987).

1.4.1 Gingivitis

Plaque-associated gingivitis is the most common form of periodontal disease. It is characterised clinically by redness, gingival bleeding, oedema, gingival sensitivity and tenderness (Löe *et al.*, 1965). Plaque development within the experimental gingivitis model is characterised by a gradual increase in mass and a thickening of bacterial plaque at the gingival margin. On analysing the composition of the bacterial plaque, it has been demonstrated that predominate bacteria are gram positive i.e. bacteria normally associated with health (Socransky, 1977). In chronic or long-standing gingivitis, 25% of the bacteria may be gram negative (van Palenstein-Helderman, 1975). Most forms of gingivitis are plaque induced, however, secondary factors appear to modify the clinical

characteristics of the disease and result in many subclassifications.

1.4.1.1 Acute necrotising ulcerative gingivitis

Acute necrotising ulcerative gingivitis is an acute, recurring, gingival infection of complex aetiology, characterised by necrosis of the tips of the gingival papillae, spontaneous bleeding pain and fetor oris (Goldhaber & Giddon, 1964; Glossary of Periodontic terms, 1986). Recurrent episodes of acute necrotising ulcerative gingivitis can develop into a chronic condition which can lead to a periodontitis type lesion (Silver, Southcott & Wade, 1974), with resultant bone loss which is characterised by marked interproximal bony craters.

1.4.1.2 Hormonally-related gingivitis

Hormonally-related gingivitis can present as pregnancy gingivitis, puberty gingivitis, gingivitis associated with birth control medication and steroid therapy (Löe, 1965; Sutcliffe, 1972; Knight & Wade, 1974; Samant *et al.*, 1976; Kalkwarf, 1978). These forms of gingivitis are characterised by an apparent exaggerated response to plaque, reflected by intense inflammation, redness, oedema and enlargement.

1.4.1.3 Drug-induced gingival overgrowth

Individuals on drug therapy i.e. phenytoin for epilepsy, cyclosporin for immunosuppression, nifedipine and other

calcium ion channel blocking drugs can directly affect the periodontal tissues, resulting in *drug-induced gingival overgrowth* (gingival hyperplasia) (reviewed by Seymour & Heasman, 1988). Clinically, the lesion starts as beadlike enlargements of the gingival margin and papilla. Enlargement continues and the marginal and papillary growths unite and excessive growth results in the formation of pseudopockets.

1.4.1.4 Desquamative gingivitis

Desquamative gingivitis is characterised by sloughing of gingival epithelium which leaves an intensely red surface. Sloughing of the epithelium is due to vesiculation and is considered a sign of disease. Lesions can be caused by allergic reactions (Perry, Deffner & Sheridan, 1973), oral manifestations of oral dermatoses - erosive lichen planus, benign mucous membrane pemphigoid, bullous pemphigoid and pemphigus vulgaris (Shklar, Meyer & Zacarian, 1969; McCarthy, & Shklar, 1980; Greenberg, 1984).

1.4.2 Periodontitis

There are four major types of periodontitis: adult, rapidly progressive, juvenile and prepubertal (Page & Schroeder, 1982).

1.4.2.1 Adult periodontitis

Adult periodontitis may have its onset in adolescence and continues for life and is not usually clinically

significant until the mid-thirties. Its severity increases with age, there is no sex bias and disease development is slow and continuous, as evaluated using pooled data (Marshall-Day, Stephens & Quigley, 1955; Russell, 1967; Löe *et al.*, 1978, 1986; Listgarten, 1986). Although microbial plaque is linked to the aetiology of periodontal disease, the overriding influence is probably individual host susceptibility. Fifteen to thirty percent of individuals are susceptible to severe periodontal destruction (Jenkins & Kinane, 1989) and the hosts inflammatory and immune processes are likely to be crucial factors in this individual predisposition.

1.4.2.2 Early-onset periodontitis

Early-onset periodontitis has been recognised for many years, but the definitions of apparently different forms of the disease are not yet clear. Subdivisions of juvenile periodontitis; into those with localised and those with generalised patterns of destruction are often made. Refractory forms of periodontitis are also recognised (McFall, 1982; Haffajee *et al.*, 1988; Van Dyke *et al.*, 1988).

The disorder is reported to include a form localised primarily to the first molars and incisors, often referred to as juvenile periodontitis; and, a more generalised form, often referred to as rapidly progressive or generalised periodontitis (Baer, 1971; Burmeister *et al.*, 1984). While

many authors refer to these disorders as distinct entities, it has been argued that they cannot be separated since both occur in the same family (Spektor, Vandesteen & Page, 1985).

Rapidly progressive periodontitis (RPP) occurs in young adult populations from their early-twenties through to mid-thirties. It is characterised by severe gingival inflammation and rapid loss of connective tissue attachment and alveolar bone support and is characterised by an acute florid inflammation of the gingival tissues. Although, the disease may not affect all the teeth, it is not confined specifically to the molar and incisor areas. Patients affected by RPP are not different from age matched population with a healthy periodontium when compared in terms of humoral immunoglobulin and complement levels (Ranney *et al.*, 1981). Also, neither the blastogenic response of blood-derived lymphocytes, nor the production of lymphocytic inhibition factor, nor tests for phagocytic or killing capacity appears to differentiate between the two groups (Tew *et al.*, 1981).

Juvenile periodontitis (JP) has its onset around puberty and is characterised by severe angular bony defects on the first permanent molars, and sometimes the incisors (Baer, 1971). The rate and severity of destruction is not consistent with the relatively sparse plaque and the lack of severe clinical signs of inflammation (Baer, 1971). JP

also appears to have a racial bias with more blacks affected than whites (Burmeister *et al.*, 1984). The onset of JP is rapid and when compared with periodontal disease in adults, the rate of attachment loss is 3 to 5 times faster and has been estimated at 4 to 5 μm per day (Waerhaug, 1977). Early stages of the disease may involve one or two sites around the first molars and incisors, and a definite diagnosis is made when at least three sites exhibit ≥ 5 mm loss of attachment. Family studies (Saxen, 1980; Saxen & Nevanlinna, 1984), strongly suggest genetic control of clinical phenotypic expression (including juvenile periodontitis and generalised periodontitis cases), but these studies, which are complicated by diagnostic limitations and age effects, have not included laboratory measures on variables known to be of importance in these disorders, namely abnormal neutrophil chemotaxis and bacterial agents (Boughman *et al.*, 1988). While the exact aetiologic relationship between altered neutrophil function and early-onset periodontal disease remains unknown, reduced neutrophil chemotaxis response has been reported frequently (Cianciola *et al.*, 1977; Lavine *et al.*, 1979; Van Dyke *et al.*, 1980; Ranney *et al.*, 1982; Ellegaard, Borregaard & Ellegaard, 1984; Page *et al.*, 1984; Suzuki *et al.*, 1984). Schenkein, Best & Gunsolley, (1991) have shown that race can influence chemotactic responses and that blacks have an increased prevalence of defects in early-onset periodontitis. Kinane *et al.* (1989a) using leading front analysis suggested

initially that a chemotactic defect existed in patients suffering from rapid onset periodontitis, but on further analysis by using a different technique, Kinane *et al.* (1989b) found no chemotactic abnormality. This suggests that the results of these conflicting studies, may not be due to intrinsic cell behaviour but may reflect the techniques used. Most studies have focused on JP, but PMN abnormalities have also been observed in generalised forms of early-onset disease (Katsuragi *et al.*, 1988). In a study of 22 families of probands with localised JP and chemotaxis defects, Van Dyke, Levine & Genco, (1985) showed that a total of 40 siblings (100% of those with localised JP) also had a neutrophil abnormality. But it is not clear whether the neutrophil abnormality creates susceptibility or if it is secondary to the actions of periodontal pathogens.

1.4.2.3 Pre-pubertal periodontitis

Pre-pubertal periodontitis (Pre-PP) is a rare condition and occurs as a generalised or localised disease (Page *et al.*, 1983; Waldrop *et al.*, 1987). Generalised Pre-PP begins with the eruption of the primary teeth and affects both primary and secondary teeth. It is characterised by severe gingival inflammation, rapid bone loss, mobility and tooth loss. PMN and mononuclear leucocyte defects are present (Page *et al.*, 1985) and work by Page, Beatty & Waldrop, (1987) suggests that the functional abnormality in PMNs may have a molecular basis. Subjects also having increased

susceptibility to skin and upper respiratory tract infection. Localised Pre-PP affects only some of the primary teeth, is less aggressive and may be related to either a PMN or mononuclear leucocyte defect, but not both.

1.4.2.4 Refractory periodontitis

Refractory periodontitis refers to disease in multiple sites in patients which continue to demonstrate attachment loss after appropriate therapy and appear to be more resistant to treatment (Lundström, Johannsson & Hamp, 1984; Haffajee *et al.*, 1988; Van Dyke *et al.*, 1988). These sites presumably continue to be infected with periodontal pathogens (Haffajee *et al.*, 1988; Van Dyke *et al.*, 1988).

1.5 Periodontal disease activity

Three models have been described to explain destruction of the periodontal supporting tissues; the *continuous paradigm*, the *random burst theory* and the *asynchronous multiple burst hypothesis* (Socransky *et al.*, 1984).

The continuous paradigm implies slow, constant and progressive destruction and is supported by cross-sectional studies (Marshall-Day *et al.*, 1955; Russell, 1967; Löe *et al.*, 1978) and longitudinal monitoring of sites not responsive to treatment (Badersten, Nilveus & Egelberg, 1985). Pooling data from groups, individuals and sites in cross-sectional studies can give the impression that destruction was slow and continuous (Löe *et al.*, 1978).

The random burst theory proposed short periods of destruction, separated by periods of no destruction, occurring randomly through time and at random sites within an individual (Socransky *et al.*, 1984). Whereas the asynchronous multiple burst hypothesis proposed that destruction occurred within a defined period of time and then the disease went into remission (Socransky *et al.*, 1984). In the multiple burst hypothesis many sites would show bursts of activity over a limited period of time and the sites would become inactive for an indefinite period.

Existing data can not refute any of these three proposed patterns of periodontal destruction and any, all or none may be relevant in any individual (Haffajee *et al.*, 1988).

1.6 Pathogenic mechanisms of periodontal disease

1.6.1 Introduction

Periodontal diseases (gingivitis and periodontitis) are bacterial infections, with plaque well documented as the causative agent (Listgarten, 1988; Christersson *et al.*, 1991). Host responses triggered by bacterial infections are immunologic and the role of the immune response in the pathogenesis of periodontal disease is still unknown. A variety of different immune effector mechanisms may be involved, also a variety of immune cells may be interacting (i.e. T and B cells, macrophages and neutrophils) and their effects could be amplified by the production of numerous enzymes, monokines and lymphokines. All these processes

may interact and cause periodontal injury, but the exact role of each cell type and mechanism within the pathogenesis of periodontal disease is still unknown.

1.6.2 Recent advances in Immunology

Immunology has developed quite rapidly in the last few years with advances in molecular genetic methodology, cloning of immunogenic cells, studies on cell surface receptors and their biological functions, regulatory mechanisms, effector mechanisms and effector mediators. Advances in these fields have been discussed extensively and the following sections will outline the basic concepts in immunology before discussing their hypothesised roles in periodontal disease.

1.6.2.1 Cells of the immune system

The immune system has evolved to protect the host against pathogenic microorganisms 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 immune response is initiated once the antigen is taken up 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.

There are two types of lymphocytes: B-lymphocytes which develop in the bone marrow and subsequently develop into antibody-producing plasma cells, and T-lymphocytes (T-cells) which develop in the thymus. T-cells can be divided into two main subsets, helper T-cells, which produce helper factors i.e. cytokines *etc* to assist other cells of the immune system or cytotoxic T-cells, which stimulate the microbicidal and cytotoxic activity of other immune cells including macrophages. Antigen is presented to T-helper cells, the activated T-helper cell then produces factors which stimulate B-cells to undergo differentiation into plasma cells and eventually produce antibodies. The factors produced by the activated T-helper cells can also stimulate T-cytotoxic cells and phagocytes, arming them for microbicidal or cytotoxic activity. The professional phagocytes include macrophages, neutrophils and various cells of the mononuclear phagocyte system which are distributed throughout the body.

1.6.2.2 Major histocompatibility complex

The success of the interaction between the T-helper cell and antigen-presenting cell is dependent upon the ability of the T-helper cell to recognise processed antigen in association with self antigen on the surface of the antigen-presenting cell. These self antigens are responsible for rejection of grafted tissue from one individual to an other, they determine if the donor tissue will be immunologically compatible with the host and hence

are called histocompatibility antigens. The major histocompatibility antigens are encoded in a set of clustered loci known as the major histocompatibility complex, and are known as the major histocompatibility complex antigen or the MHC. MHC as well as playing a major role in graft rejection also plays a very important role in antigenic peptide recognition. T-lymphocytes have to recognise foreign antigens in association with MHC i.e. MHC-restricted antigen recognition. In humans MHC is called human leucocyte antigen complex, or the HLA complex, which is encoded on gene complexes found on chromosomes 6 and 15. The HLA is divided into two major classes I and II; each of the classes has two subclasses, alpha and beta; which are further divided into families and sub-families which are designated by capital letters. In humans the MHC class II alpha families are DP, DQ, DR and DN; and the MHC class II beta families are designated DO, DP, DQ, and DR. The human class II, subclass alpha families are designated DPA-1, DPA-2, *etc*; whereas in class II, subclass beta families are designated DRB-1, DRB-2, *etc*. In general, MHC class I antigens are found on most nucleated cells and MHC class II antigens are found mainly on activated T-cells and on B-cells.

1.6.2.3 T-cell receptor

When an antigen-presenting cell presents antigen to a T-cell, the T-cell simultaneously binds the antigen and the MHC molecule on the antigen-presenting cell, this allows

differentiation between self and non-self. All T-lymphocytes express a receptor known as the T-cell receptor, which binds antigens and MHC molecules specifically. Two types of T-cell receptor exist the αβ heterodimer and the γδ heterodimer; with the αβ 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, B-lymphocytes possess the ability to recognise antigen alone.

1.6.2.4 Cluster of differentiation (CD) antigens

CD antigens are a family of cell surface molecules which were discovered by immunising rabbits, mice *etc* with human lymphocytes. The antibodies produced recognise unique antigens designated CD antigen (Cluster of differentiation). These CD antigens are useful for differentiating leucocyte populations, for example, the monoclonal CD3 is useful for distinguishing T-cells from B-cells; CD4 is present on T-helper cells and is the co-receptor for class II MHC and is also a receptor for the human immunodeficiency virus; CD8 antigen is present on T-cytotoxic cells and is the co-receptor for class I MHC antigen complexes. Therefore, $CD4^+$ T-helper cells recognise antigen in a MHC class II restricted fashion and $CD8^+$ cells recognise antigen in a MHC class I restricted fashion.

1.6.2.5 T-cell subsets

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 based on their expression of distinct isoforms of the common leucocyte antigen, the CD45 molecule (Tedder, Clement & Cooper, 1985a; Tedder, Cooper & Clement, 1985b; Clement, Yamashita & Martin, 1988; Sanders *et al.*, 1988; Serra *et al.*, 1988; Salmon, Kitas & Bacon, 1989; Yamashita & Clement, 1989). T-lymphocytes that express on their surface high molecular mass CD45 isoform (the CD45RA molecule) (2H4) (Streuli *et al.*, 1988), include those lymphocytes which are unable to respond to previously encountered antigen (Morimoto *et al.*, 1985; Smith *et al.*, 1986; Damle, Childs & Doyle, 1987) and these $CD45RA^+$ T-lymphocytes are postulated to represent a pool of virgin or naive T-lymphocytes that have yet to encounter their respective antigen (Damle *et al.*, 1987). T-lymphocytes that express on their surface the low molecular mass CD45 isoform, the CD45RO molecule (Streuli *et al.*, 1988), but not the CD45RA molecule, include T-lymphocytes that have the ability to respond to previously encountered antigen. Hence the $CD45RO^+$ T-lymphocytes are thought to represent a pool of primed or memory T-lymphocytes (Smith *et al.*, 1986; Damle *et al.*, 1987; Akbar *et al.*, 1988; Yamashita & Clement, 1989) and is recognised by the UCHL-1 monoclonal antibody (Smith *et al.*, 1986). T-lymphocytes within these two mutually exclusive and maturationally distinct subsets

are also known to possess distinct activation requirements and also distinct profiles of lymphokine production (Byrne, Butler & Cooper, 1988; Sanders *et al.*, 1989; Salmon *et al.*, 1988, 1989).

1.6.2.6 The immune response

Generally cell mediated immunity is initiated when antigen from subgingival plaque penetrates into the connective tissue through the junctional epithelium. Antigen presenting cells (macrophages and LCs) process the antigen and alter it to a form recognizable by the immune system i.e. an antigenic peptide in association with class II MHC. The T-helper cell ($CD4^+$) recognises this association of foreign antigen and self MHC and becomes stimulated, proliferates and releases cytokines. The cytokines in turn then act on other lymphoid cells (ie macrophages, B-cells and other T-cells) to produce tissue damage, inflammation and bone resorption. The lymphocytes are now sensitised, upon re-exposure to the plaque antigens they respond by proliferating and synthesising cytokines. The cytokines then act as signals which stimulate, inhibit or even kill other cell types. This is similar to the delayed type hypersensitivity lesions where in addition to sensitised lymphocytes, the lesions contain non-sensitised lymphocytes, which probably form part of the inflammatory cell infiltrate in periodontal disease. They may also produce cytokines when stimulated by mitogenic substances

released either by the subgingival microbiota or by other cells in the inflammatory reaction.

1.6.3 Damage caused by plaque microorganisms

Most forms of human periodontal disease are initiated and sustained by factors produced/released by subgingival microbiota (Kahnberg, Lindhe & Hellden, 1976). Some of these substances can damage host cells and tissues directly and others indirectly by activating endogenous cellular and humoral inflammatory reactions. It is therefore the combination of both direct and indirect pathways that account for periodontal injury.

Plaque microorganisms can directly damage cellular and structural components of the periodontium via microbial invasion of the soft tissues, as well as the formation of noxious substances by the microbiota of the gingival pocket. The release of soluble irritative substances by bacteria could cause disease, Taichman *et al.*'s (1984) group isolated a 'leukotoxin' from *Actinobacillus actinomycetemcomitans* which they found to destroys neutrophils and monocytes. Depressing the first line of defence of the immune system within the gingival tissue and in theory increases the hosts susceptibility to periodontal disease. This finding that deficient numbers of PMNs may increase susceptibility to periodontal disease is supported by the studies carried out on experimentally induced neutropenia (Attström & Schroeder, 1979; Schroeder &

Attström, 1979), and also that individuals with underlying neutrophil defects have increased susceptibility to periodontal disease (Genco *et al.*, 1986). The leukotoxin isolated by Taichman *et al.* (1984) has yet to be isolated by other investigators, and its role further investigated. Also, some bacterial hydrolytic enzymes can attack various components of the intercellular matrix of the epithelium and connective tissue (ie collagen, elastin, fibronectin and fibrin), these factors could impair gingival anti-bacterial defense.

Indirect injury by plaque microorganisms can occur via inflammation. Many microbial products have little or no direct toxic effect on the host, instead they possess the potential to activate non-immune and/or immune inflammatory reactions which in turn actually cause the damage. For instance lipopolysaccharide (LPS) (endotoxins) of gram negative microorganisms may be responsible for a spectrum of inflammatory events because they interact with and modulate the behaviour of leucocytes. Endotoxin can act as a mitogen, stimulating B cell proliferation and antibody secretion, it can also cause macrophages to produce and secrete neutral proteinases and acid hydrolases. It promotes osteoclastic bone resorption and also has profound effects on the blood coagulation system. Further, endotoxin triggers the complement system resulting in the formation of numerous inflammatory peptides. These are only a few of the biological properties of endotoxins and

most of these properties are shared by lipoteichoic acids from gram positive bacteria. Likewise, specific proteins and polysaccharides produced and released from several subgingival bacteria activate endogenous mediators of vascular permeability, encourage inflammatory cells to move into the tissue and cause them to discharge pro-inflammatory agents.

Many members of the gingival microbiota induce an immune response in the host (Seymour, 1987) and these immune reactions to antigens derived from the subgingival microbiota can be harmful to the host. One of the first signs that defense mechanisms are acting against the bacteria colonising the teeth, is the swelling of the vessels of the gingival margin (Egelberg, 1967). This is followed by an increased vascular permeability, exudation of gingival fluid and migration of PMNs into the gingival sulcus, resulting in the tissues becoming inflamed.

The gingival fluid contains both complement factors and specific antibody. Two pathways of complement activation exist. The classical pathway of complement becomes activated when specific antibodies of IgG or IgM classes react with bacteria, whereas the alternative pathway of complement is activated by various microbial cell wall constituents such as LPS, lipoteichoic acids and polysaccharides.

1.6.4 Leucocytes within gingival tissue

Both polymorphonuclear and mononuclear leucocytes take part in the phagocytic defense of the dentogingival area and this defense is presumably assisted by the presence of complement and often specific antibodies. In order to be effective 'defenders' the phagocytes have to recognise the site of microbial invasion, they have to leave the blood vessels and migrate through the tissue to the site of invasion, where they have to ingest and kill the microorganisms (Van Dyke *et al.*, 1985). The leucocytes also have a microbicidal system that works under anaerobic conditions (Rice *et al.*, 1987) which may be crucial in the gingival crevice. The products released by the leucocytes not only kill invading microorganisms but the oxygen products and the lysosomal proteases may also cause extensive host tissue injuries (Fantone & Ward, 1982).

PMNs are the predominate leucocyte found within the gingival crevice (Skapski & Lehner, 1976; Sigusch *et al.*, 1992) and appear to be phagocytic and microbicidal cells in healthy or moderately inflamed sites (Wilton, 1982, 1984), respond to external stimuli (Farber, 1989) and are potentially able to participate in the pathophysiology of periodontal disease. The possible contribution of PMNs to gingival inflammatory injury has been thoroughly investigated (Taichman *et al.*, 1984) and it has been shown that as PMNs leave the circulation and move through the tissues, they are probably constantly secreting pro-

inflammatory agents into the environment (Freedman *et al.*, 1968; Benedek-Spät *et al.*, 1991; Miyasaki, 1991; Altman *et al.*, 1992; Giannopoulou *et al.*, 1992). Under experimental conditions it has been shown that PMN reactions do contribute to gingival injury, for example PMNs supernates can cause epithelial cell detachment *in vitro* (Altman *et al.*, 1992); topical application of PMN lysosomal extracts onto the gingival margin promotes an increase in vascular permeability and leucocyte migration in experimental animals (Kahnberg & Hellden, 1977); short-term reduction of circulating neutrophils (by cytotoxic drugs or anti-neutrophil antiserum) is accompanied by a decrease in the clinical signs of gingivitis in dogs (Attström, Tynelius-Bratthall & Egelberg, 1971; Rylander, Attström & Lindhe, 1975; Attström & Schroeder, 1979) and finally intradermal injections of plaque produced more local tissue damage in normal as compared to neutropenic rabbits (Taichman, Freedman & Uriuhara, 1966). It would therefore appear that PMNs can contribute to gingival injury.

Patients with neutropenia, in which the number of cells is markedly depressed (below 1500 per µl of peripheral blood) (Finch, 1983), present with a variety of periodontal manifestations. In the malignant form (agranulocytosis, PMN count below 500 per µl), which is generally drug induced, there is ulceration and necrosis of the marginal gingiva. This is associated with bleeding, and occasional involvement of the attached gingiva (Awbrey & Hibbard,

1973). Histologically, the ulcerated areas exhibit no PMN response. In more protracted forms of the disease such as cyclic, chronic and familial benign neutropenia the lesions are frequently severe. The gingivae may be oedematous, hyperaemic and hyperplastic with areas of partial desquamation (Kyle & Linman, 1970; Baer & Benjamin, 1974). These features are often accompanied by deep periodontal pockets and extensive, generalized bone loss involving the permanent dentition (Levine, 1959; Baehni *et al.*, 1983). Occasionally bone resorption may involve the deciduous dentition (Cohen & Morris, 1961; Lampert & Fesseler, 1975). Despite the obvious decrease in host defences caused by neutrophil depletion in familial benign chronic neutropenia, periodontitis is not always inevitable (Deasy *et al.*, 1980). Deasy *et al.* (1980) describe a family exhibiting familial benign chronic neutropenia and show that although several individuals within this family were neutropenic, not all were affected either by recurrent infections or by periodontal disease. One neutropenic sibling who was free from periodontal disease, had good oral hygiene, whereas her neutropenic brother, with poor oral hygiene had fiery-red oedematous gingiva and early generalised periodontitis. These findings might be explained by the variable expressivity of the disorder between the siblings, or on the interaction of the environment (e.g. the oral hygiene) on this disorder.

Most of the substances produced and released by plaque microorganisms are antigenic, eliciting both cell-mediated and humoral mediated immune reactions. Systemic and local antibody synthesis by lymphoid cells may be more relevant in gingival defense than the systemic immune response, as it has been shown that gingival exudation usually contain higher concentrations of antibody directed against specific plaque organisms when compared to serum, producing an enriched antibody environment in the immediate vicinity of the infection (Lally *et al.*, 1980; Tew *et al.*, 1985).

Following the changes in permeability, the activation of complement and the influx of polymorphs, the last arrivals at the site of inflammation are the mononuclear cells - lymphocytes and monocytes. Lymphocytes are usually specific in their attack and only cause harm when they are activated non-specifically. In addition, monocytes and macrophages are equipped with enzymes normally used in the process of mopping up dead tissue cells and polymorphs, but which can also damage healthy cells including other macrophages.

1.6.5 Cell mediated immune responses within the gingiva

The role of T-lymphocytes in gingivitis and periodontitis has been investigated (Seymour *et al.*, 1982; Daly, Clancy & Cripps, 1983a; Okada *et al.*, 1984; Gillet *et al.*, 1986; Johannessen *et al.*, 1986; Stoufi *et al.*, 1987; Meng & Zheng, 1989; Afar, Engel & Clark, 1992).

1.6.5.1 Experimental gingivitis

The experimental gingivitis model was first introduced by Löe *et al.* (1965). They demonstrated that withdrawal of oral hygiene led to increased plaque levels which subsequently led to gingival inflammation. Removal of plaque results in resolution of the inflammation, with no long term deleterious effects. The experimental gingivitis model is thus an appropriate model to use when the initiation of gingival inflammation needs to be studied under experimental conditions. Some studies have utilised the experimental gingivitis model to elucidate the role of T-cells within the developing inflammatory reaction. Histologically normal gingival tissue is only found adjacent to relatively plaque-free teeth and is therefore relatively rare in humans. Biopsies of clinically healthy human gingiva contains inflammatory cells consisting of predominantly T-cells with few B cells or plasma cells (Page & Schroeder, 1976; Seymour *et al.*, 1983a, 1983b; Joachim *et al.*, 1990). Four to seven days of plaque accumulation results in an influx of lymphocytes into the tissue (Listgarten & Ellegaard, 1973; Payne *et al.*, 1975) and after three weeks of plaque accumulation the lesion is mainly T-cell dominated (Seymour *et al.*, 1983a, 1983b; Brecx *et al.*, 1988b). After 6 months of plaque accumulation the lesions are still dominated by lymphocytes and PMNs (Brecx *et al.*, 1988a). Plasma cells have occasionally been observed in the initial and early lesion of the experimental gingivitis model (Brecx *et al.*, 1987b,

1988b), but greater than six months of plaque accumulation appears to be a prerequisite for plasma cells to constitute a significant proportion of the infiltrated connective tissue (Brecx *et al.*, 1988a). Plasma cells have only been found to be the major component of infiltrated connective tissue in the advanced lesion where bone loss is evident. In general, within chronically inflamed tissue the numbers of plasma cells do appear to increase with severity of the lesion (Ogawa *et al.*, 1989).

1.6.6 T-suppressor cell and T-helper cell ratios

In adult periodontitis only about 30% of the infiltrating cells are T-lymphocytes (Okada *et al.*, 1984), with increasing numbers of T-helper cells seen (increased T helper:suppressor ratio); in juvenile and rapidly progressing periodontitis patients have decreased peripheral blood helper to suppressor cell ratios whilst the disease is active (Kinane, Johnson & Evans, 1989). However, a range of helper:suppressor T-cell ratios from low to high have been reported in patients with rapidly progressive periodontitis (Katz *et al.*, 1988). Low T helper:suppressor (T4:T8) has been linked to redness and swelling of the gingiva (Stashenko *et al.*, 1985) and also a correlation between probeable pocket depth and T4:T8 ratio has been demonstrated (Stoufi *et al.*, 1987). Depressed T helper to suppressor ratios in juvenile and rapidly progressive periodontitis may represent abnormal T-cell regulation of the immune response and may explain the

rapid breakdown seen within these disease classifications. In comparison adult periodontitis expresses elevated ratios, suggesting 'over-regulation' of the immune response which may explain the slower rate of disease development when compared with juvenile and rapidly progressive periodontitis. But the significance of a T4:T8 balance in periodontal disease is still unknown (Meng & Zheng, 1989).

It has been hypothesised, that chronic adult periodontitis can be separated into two stages, quiescent or activated. It is suggested that the quiescent stage is B cell dominated whereas the activated stage is plasma cell dominated (Gillet *et al.*, 1986). Active periodontal sites appear to have an increased number of B cells with a decreased T:B cell ratio when compared with stable/healthy sites (Reinhardt *et al.*, 1988), but no significant differences in T4:T8 ratio could be demonstrated between the two sites. Before trying to attempt to identify the type of cell present within the mononuclear infiltrates of diseased gingival tissue, investigators should try to determine whether the site is active or inactive before grouping them together. If the assumption is made that the sites are of similar disease state, then the results of the type of cell present within the infiltrate may be false, as it has been noted by Saglie *et al.* (1988), that different cell types appear to be elevated in different disease sites i.e. active sites appeared to be associated with few T helper/inducer cells and macrophages with increased T

suppressor/cytotoxic cells, B cells and Langerhans cells, whereas sites of inactivity tended to have increased T helper/inducer cells, NK cell, monocytes and have decreased T suppressor/cytotoxic cells (Saglie *et al.*, 1988).

Recent work, has suggested that depressed spontaneous lymphocyte proliferation is seen in subjects with periodontal disease; and this decrease is due to altered T-cell control. Amer *et al.* (1990) demonstrated that separated B cell populations have significantly higher proliferative responses than unseparated mononuclear cells within the patient groups only. This suggests that T-cells were reducing the hyper-responsiveness of the B-cells (Amer *et al.*, 1990) and that disturbances in the immunoregulation within the periodontium exist. This study used very few subjects and in comparison to other studies the suppression of the spontaneous lymphocyte proliferation seen does not reach the levels of the other studies (Tew *et al.*, 1983). It has also been suggested that this dysfunction is confined to the periodontium as no difference in the peripheral blood lymphocyte subpopulation could be demonstrated when healthy individual were compared with individuals suffering from chronic adult periodontitis (Zafiropoulos *et al.*, 1990). This suggests that longitudinal studies and follow up examinations (locally and in peripheral blood) of patients suffering from periodontal disease at different stages of disease are

necessary to ascertain the true role of immunoregulation dysfunction within the periodontium.

1.6.7 Memory T-cells and Naive/virgin T-cells

T-lymphocytes play a crucial role during the initiation and maintenance of chronic inflammatory responses by releasing cytokines and other helper factors to propagate and maintain the immune response. Perivascular accumulation of T-lymphocytes with the $CD29^+$ $CD45RO^+$ memory phenotype (Parrott & Wilkinson, 1981) and localised increase in vascular endothelium permeability at the site of chronic inflammation (Poulter *et al.*, 1985; Hemler *et al.*, 1986) are two of the commonly observed events associated with chronic inflammatory responses.

It has been demonstrated that memory T-lymphocytes ($CD29^+$ $CD45RO^+$) adhere to vascular endothelial cells and augment their permeability to macromolecules (Damle & Doyle, 1990). This property of memory T-lymphocytes to modulate endothelial permeability may be a dominant factor contributing to preferential migration of memory T-cells into sites of chronic inflammation. Numbers of memory T-cells have also been shown to be increased in the skin of patients with systemic sclerosis (Rustin *et al.*, 1990); accumulation of memory T-cells to sites of inflammation may be explained by their difference in ability to adhere to endothelial cells and their homotypic adhesion (Pitzalis *et al.*, 1988).

Recently Afar *et al.* (1992) have demonstrated elevated levels of memory T-cells ($CD45RO^+$) in the peripheral blood of patients suffering from periodontal disease whereas in contrast Walsh *et al.* (1989) found that in Oral Lichen Planus the expression of the naive T-cell marker - $CD45RA^+$ was confined to the intraepithelial lymphocytes (IELs), suggesting that they may play an immunoregulatory role in T-cell responses. It has been hypothesised that switching from the naive ($CD45RA^+$) to memory ($CD45RO^+$) does occur and that this switching is linked to the maturation and activation state of the T-cells, with an intermediate $CD45RA^+$ $CD45RO^+$ T-cell (Wallace & Beverly, 1990). This suggests that stimulated memory T-cells may play a role in *in vitro* recruitment of naive T-cells.

Abnormal distribution of T-lymphocyte subsets occurs within synovial fluid when compared with peripheral blood (Pitzalis *et al.*, 1987). A virtual absence of naive/virgin T-cells ($2H4^+$) was noted, with increases in the T-helper/inducer ($4B4^+$) and memory T-cells ($UCHL\text{-}1^+$). The exact role of memory T-cells and naive/virgin T-cells in any disease state is not known i.e. in rheumatoid arthritis (Pitzalis *et al.*, 1987); multiple sclerosis (Morimoto *et al.*, 1987) *etc* and especially within periodontal disease.

Other studies demonstrate that periodontal patients have circulating lymphocytes which are sensitized to substances originating from subgingival plaque organisms (Ivanyi &

Lehner, 1970; Lang & Smith, 1977; Reed, Neiders & Genco 1976), though these could be cross-reacting. Some experiments seem to suggest that a correlation exists between the severity of the periodontal disease and the number of sensitised lymphocytes i.e. as demonstrated by Malberg *et al.* (1992) that as gingival index increased so did the number of activated $CD4^+$ cells; however, this association requires confirmation by other studies. It has also been suggested that periodontal lesions may contain subsets of T-cells which have been sensitised and are responding to different plaque antigens (O'Neill, Woodson & Mackler, 1982; O'Neill & Woodson, 1986).

1.6.8 Macrophages within the gingival tissues

The participation of macrophages is crucial to the development of cell-mediated hypersensitivity reactions. Upon being recruited into the inflammatory lesions, macrophages become activated under the influence of lymphokines and other substances, such as endotoxin and bacterial cell walls. Activated macrophages are more adherent, phagocytose and kill bacteria more efficiently and they also acquire cytotoxic properties enabling them to attack and destroy the target cells. Activated macrophages synthesis and secrete numerous products which have wide-ranging biological potentials. These include hydrolytic enzymes, complement components, binding components (fibronectin), oxygen metabolites, arachidonic acid metabolites and monokines (e.g. IL-1). Lysosomal enzymes,

collagenase and elastase probably facilitate extracellular breakdown of the connective tissue matrix while prostaglandins and monokines may have a number of effects on the growth and activity of other cells, including T and B lymphocytes, fibroblasts and osteoclasts.

Macrophage numbers over the three week experimental gingivitis period have been shown by some investigators not to change significantly in the percentage of cells present (Brecx *et al.*, 1987b, 1988a; Seymour *et al.*, 1988). However, Topoll *et al.* (1989) analyzed subsets of macrophages over a 19-day period of experimental gingivitis. They found that there was no overall changes in the relative numbers of total macrophages present over the 19-day period, but a change in the type of macrophage present had occurred i.e. the number of anti-inflammatory macrophages had decreased with a simultaneous increase in the number of inflammatory macrophages. Inflammatory macrophages, are antigen elicited macrophages which process and present antigen efficiently resulting in the initiation of an inflammatory/immune response. Other investigators have shown that mononuclear phagocytes invariably appear in association with osteoclasts at sites of active bone resorption (Rifkin & Heijl, 1979) and that endotoxin-stimulated mononuclear phagocytes secrete soluble factors with bone resorbing activity (Gregory, 1988). This suggests that macrophages play a role in the pathogenesis of periodontal disease.

1.7 Langerhans cells

LCs are dendritic, suprabasal cells found in most stratified squamous epithelia including epithelia of the oral mucosa (Daniels, 1984). They are of bone marrow origin (Katz, Tamaki & Sachs, 1979) and share many characteristics in common with the macrophage (Thorbecke, Silberberg-Sinakin & Flotte, 1980). LCs express Fc IgG receptors, C3 receptors (Stingl *et al.*, 1977) as well as CD1 and Class II MHC antigens (Klareskog *et al.*, 1977; Rowden, Lewis & Sullivan, 1977) and are capable of antigen presentation (Silberberg-Sinakin *et al.*, 1980; Walsh, Seymour & Powell, 1988; Cruchley *et al.*, 1989). The important role of the gingival LC as accessory cells in mitogen induced T-cell responses has been demonstrated by Newcomb & Powell, (1988) as has their important function as antigen gathering and presenting cells (Rowden, 1981; Newcomb & Powell, 1988).

LCs in the oral mucosa are present in numbers equivalent to those found in skin (Ahlfors, Larsson & Bergstresser, 1985; Van Loon, 1989). Sensitization of Langerhans cells may occur via antigens seeping through the gingival mucosa and in theory, these sensitised Langerhans cells could present antigen to T-helper cells within the gingiva initiating an immune response. Other investigators, have shown that epithelium resident human Langerhans cells are similar to immature precursors of lymphoid dendritic cells found in skin-draining lymph nodes (Romani *et al.*, 1989). Oral Langerhans cells can function as antigen

presenting cells in a way similar to the skin Langerhans cells which present antigen in contact hypersensitivity (Silberberg-Sinakin *et al.*, 1980).

It has been proposed that the LCs present within gingival oral epithelium vary in number due to the different bacterial stimulation levels, or alternatively different responses are seen to similar plaque antigens penetrating the surface of the oral epithelium (Baelum, Fejerskov & Dabelsteen, 1989). These cells increase in number as plaque accumulates (Newcomb, Seymour & Powell, 1982; Difranco *et al.*, 1985); are increased in inflamed tissue; and their numbers are increased in oral epithelium found to contain intragingival microorganisms (Saglie *et al.*, 1988). In clinically inflamed tissues and in experimentally induced gingivitis the LCs morphology appears to be more dendritic (Newcomb *et al.*, 1982; Difranco *et al.*, 1985) and have increased numbers of granules (Newcomb & Powell, 1986a). Inflammation increases Class II MHC expression on LCs and this facilitates the LC functional role by permitting MHC-restricted antigen presentation to T-helper cells (Savage, Walsh & Seymour, 1987).

Interferon-γ (IFN-γ) produced by activated T-lymphocytes can induce differential expression of Class II MHC, it induces HLA-DR and DQ on LCs and only HLA-DR on keratinocytes (Walsh, Seymour & Powell, 1986a), in addition LCs express more DR than DQ (Walsh, Seymour & Powell,

1987b). The functional significance of differential expression of DR and DQ in gingival epithelium is very likely linked to the central roles of class II products in regulation of the immune response (Becker, 1985). The main restriction elements for antigen presentation are located on DR molecules, while DQ molecules play a minor role (Gonwa *et al.*, 1983; Walsh *et al.*, 1986a). It has been established that a DQ^+ subpopulation of peripheral blood monocytes contain the entire antigen-presenting capability of the monocyte population (Gonwa *et al.*, 1983). While both DR and DQ are important in antigen presentation by macrophages (Walsh *et al.*, 1986a), further studies utilising various LC subpopulations are required to establish the role of these molecules in Langerhans cell function.

IL-1 produced by epithelial cells (Mergenhagen, 1984) augmented through epithelial cell injury increases CD1 expression on gingival LCs precursors (Walsh *et al.*, 1986a; Walsh, Seymour & Powell, 1986b). CD1 is a family of cell surface glycoproteins of unknown function expressed on immature thymocytes, epidermal LCs and on a subset of B-lymphocytes. Increased CD1 expression on gingival LCs may be important, as a role for CD1 molecules has been suggested in antigen recognition. Due to structural similarities of CD1 to the MHC molecule of known antigen presenting cells. It has been suggested that some peripheral T-cells can recognise LCs CD1 molecules in

association with an antigenic peptide (instead of MHC) and that this recognition triggers cellular events similar to those seen when T-cells interact with antigen presented by self-MHC molecules (Porcelli *et al.*, 1989). It is likely that these $CD1^+$ LCs may interact directly (Moulon, Peguet-Navarro & Schmitt, 1991) or through macrophages in T-cell activation.

Walsh *et al.* (1988) hypothesise that epithelial cells produce IL-1 which acts on an intraepithelial population to transform these cells from $CD1^-$ to $CD1^+$. LPS and/or IFN-γ then acts on these $CD1^+$ cells making them at first DR^+ and finally DQ^+ as well. It is hypothesised that these cells ($CD1^+$ $HLA\text{-}DR^+$ $HLA\text{-}DQ^+$) could then act as antigen presenting cells. CD1 is a glycoprotein detectable on the surface of LCs (Murphy *et al.*, 1981). The OKT6 monoclonal antibody recognises CD1a on the surface of LCs and is the most sensitive method available at the present time for detecting LCs (Harrist *et al.*, 1983; Difranco *et al.*, 1985).

Hitzig *et al.*, (1989) reported a cross-sectional study where gingival tissue was categorised according to clinical indices. These authors suggested LC numbers increase during early stages of inflammation and decrease in severe inflammation. These findings are in contrast to longitudinal studies where LC numbers were reported to increase steadily with gingival inflammation (Newcomb *et*

al., 1982; DiFranco *et al.*, 1985; Newcomb & Powell, 1986a, 1986b).

1.8 Cytokines

1.8.1 Introduction

The first evidence that soluble factors released by cells were involved in immune reactions and might affect the accumulation of mononuclear cells in cellular hypersensitivity reactions was provided by Bloom & Bennet, (1966) and by David, (1966). These investigators independently described a soluble protein factor designated migration inhibitory factor, believed to be derived from lymphocytes, that inhibited macrophage migration from capillary tubes *in vitro*. Later Dumonde *et al.* (1969) used the term *lymphokine* to describe such lymphocyte-derived hormone-like substances. Similar substances produced by monocytes became known as *monokines*. When it was discovered that cells other than those of the immune system could also secrete these protein mediators they became known collectively as *cytokines* (Cohen, 1986).

Cytokines are a heterogeneous group of protein cell regulators variously called lymphokines, monokines, interleukins and interferons that have in common a number of features:

(1) Cytokines are soluble, secreted proteins of low molecular weight (<80 kDa) and are frequently glycosylated;

(2) They are involved in inflammation and immunity where they regulate the amplitude and duration of the response;
(3) They are usually produced locally and transiently and act in an autocrine or paracrine fashion;
(4) They are extremely potent and act by binding specific cell surface receptors on the target cell.

The involvement of cytokines in the inflammatory response *in vivo* was supported by their detection in the inflammatory exudates of delayed type hypersensitivity reactions (Cohen *et al.*, 1973), the synovial fluid and culture supernatants of synovial tissue from rheumatoid patients (Stastny, 1975) and in the lymph draining areas of antigenic challenge (Kelly, 1972; Hay, Lachman & Trnka, 1973). Early studies of cytokine action were largely performed using crude tissue extracts or culture supernatants. More recently the availability of purified and recombinant cytokines has greatly facilitated their study and has resulted in an enormous increase in both the literature and our understanding of their actions *in vivo*. It is however useful to consider the cytokines studied in the proceeding chapters i.e. interleukin 1 (IL-1), tumour necrosis factor (TNF) and interferon (IFN), in more detail.

1.8.1.1 Interleukins

The name 'interleukin' was proposed in 1979 for factors produced and released by activated T-lymphocytes that act on other lymphocytes to produce biological effects i.e. the

interleukins act as intercellular signalling agents between lymphocytes. Interleukins also include species produced primarily by cells of other hematopoietic lineages, such as monocytes. Interleukin-1 (IL-1) is a monokine (Platanias & Vogelzang, 1990), produced by monocytes in response to fever-inducing agents such as bacterial lipopolysaccharide (LPS). It was previously known as 'lymphocyte activating factor' or 'endogenous pyrogen' because of its fever-inducing properties.

IL-1 is a pleiotropic cytokine with diverse activities including induction of thymocyte proliferation, PMN chemotaxis, fever, fibroblast mitogenesis and collagenase synthesis (Dinarello, 1988; Jandinski, 1988). IL-1 can be found in two forms, IL-1α and IL-1β (March *et al.*, 1985). The predominant form secreted by monocytes is IL-1β, in contrast, human keratinocytes also express both IL-1α and IL-1β mRNA, but appear to produce mostly IL-1α (Kupper *et al.*, 1986). Since macrophages and epidermal cells are components of periodontal tissue, these cell types may be a significant source of IL-1.

1.8.1.2 Tumour necrosis factor

Tumour necrosis factor (TNF) is functionally related to IL-1. Two forms have been detected TNFα and TNFβ, which are products of activated macrophages and lymphocytes respectively. TNFα was originally discovered in the sera of mice and rabbits that had been infected with *Mycobacterium*

bovis strain bacillus Calmette-Guérin and subsequently with endotoxin (Carswell *et al.*, 1975). Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice. TNFβ (also called lymphotoxin) was first characterised as a biological activity in mitogen-stimulated lymphocytes, having anti-cellular action on neoplastic cell lines (Charon *et al.*, 1982a). Although originally defined by their cytotoxic action, it has become apparent with the availability of recombinant TNFα, that the TNFs are potent immunoregulatory molecules sharing overlapping biological activities with IL-1. Furthermore, several lines of evidence point to the existence of bidirectional stimulatory interactions between TNFα and IL-1 (Le & Vilcek, 1987).

1.8.1.3 Interferons

The name interferon (IFN) was given to a substance, isolated by Isaacs & Lindenmann in 1957, that protected cells against viral infection. Three types of IFN exist, α, β and γ. Type I IFNs, IFNα and IFNβ and are produced by leucocytes and fibroblasts respectively; and type II IFN, IFNγ is known as immune IFN. IFNγ is produced by lymphocytes following antigenic or mitogenic stimulation and is acid-labile. One of the main functions of IFNγ is macrophage activating factor, i.e. IFNγ can act on tissue macrophages found in skin, Langerhans cells; in bone, osteoclasts; in liver, Kupffer cells and in the central

nervous system, microglial cells. IFNs are potent regulators of MHC antigens, with IFNγ by far the most effective inducer of *de novo* synthesis of MHC class II (HLA-DR, HLA-DP and HLA-DQ) antigens. Thus, the effect of IFNγ stimulation on macrophages is to increase the expression of both MHC class I and class II antigens, class I being required for recognition of foreign antigens by cytotoxic T-lymphocytes and class II being required for recognition of foreign antigens by T-helper cells. IFNγ has also been shown to increase adhesion molecule expression on endothelial cells to facilitate cellular migration (Yu *et al.*, 1985; Dustin *et al.*, 1988; Thornhill & Haskard, 1990); and has also been detected in GCF from chronic adult periodontitis patients who have exhibited ≥ 2mm clinical attachment loss (Grbic *et al.*, 1991). This suggests that IFNγ may play a role in the pathophysiology of periodontal disease.

1.8.2 Bone resorbing cytokines

The cytokines IL-1α, IL-1β and TNF-α stimulate bone resorption (Gowen *et al.*, 1983; Dewhirst *et al.*, 1985; Bertolini *et al.*, 1986; Stashenko *et al.*, 1987) and inhibit bone formation *in vitro* (Bertolini *et al.*, 1986) and *in vivo* (Sabatini *et al.*, 1988).

IL-1α, IL-1β and TNFα activity has been studied in GCF samples from clinically inflamed sites in humans (Charon *et al.*, 1982a; Hönig *et al.*, 1989; ; Masada *et al.*, 1990;

Rossomando, Kennedy & Hadjimichael, 1990; Wilton *et al.*, 1992). Further studies have attempted to localise the cytokines in healthy and diseased tissue (Jandinski *et al.*, 1991; Stashenko *et al.*, 1991a, 1991b). These studies suggest that IL-1β is more important than TNFα, which in turn is more important than IL-1α, in affecting bone resorption. Studies on the mechanism of IL-1β action on fibroblasts *in vitro*, suggests that IL-1β can act on the fibroblasts to promote cellular matrix repair (Bartold, 1988) or destruction (Mochan, Armor & Sporer, 1988; Richards & Rutherford, 1990). Human gingival lymphocytes produce cytokines (O'Neill & Woodson, 1986) and these cytokines, IL-1, TNF and GM-CSF (granulocyte macrophage colony stimulating factor), are able to promote IL-1β gene expression in human peripheral blood PMNs (Marucha, Zeff & Kreutzer, 1991). It has also been shown that dental implant material can promote peripheral mononuclear cells to produce IL-1β and TNFα which subsequently, in theory, results in bone loss and loss of the implant, though the clinical implications are not so clear (Perala *et al.*, 1992).

It has been hypothesised that periodontal pathogens may mediate connective tissue degradation in inflammatory periodontal diseases through the ability of antigens from their cell walls to stimulate IL-1 and TNFα production by circulating mononuclear cells (Meikle, Heath & Reynolds, 1986). These in turn induce the synthesis of the matrix

metalloproteinases (MMPs) collagenase, gelatinase and stromelysin by resident gingival cells, there by initiating matrix degradation (Meikle *et al.*, 1989). Bacterial plaque initiates the immune response (Newman, 1990), and its continued presence is essential for the maintenance of the inflammatory state. The host response is now driven by a complex network of cytokines produced by the lymphocytes, macrophages and other cells of the inflammatory infiltrate which perpetuate and amplify the process. With tissue damage resulting more from the side effects of these mediators on the constituent cells of the periodontium, than to the direct action of bacteria and/or their products.

1.9 Interaction between endothelial cells and leucocytes

1.9.1 Introduction

The endothelium which lines the vasculature, structurally and functionally separates the blood elements from extravascular tissue. During a local inflammatory response, injury to a tissue site results in release of chemical mediators of inflammation which change vasculature proteins and cells of the blood accumulate at the site of the injury; and localised adhesion of peripheral blood leucocytes to the endothelial cell lining occurs. Adhesion of leucocytes is an essential step in a variety of pathophysiological processes and a key event in the pathogenesis of certain vascular diseases. Cellular migration involves three main structures; these are the

endothelial cells, the cell adhesion molecules (receptors and their ligands) and the extravasating cells.

1.9.2 The endothelial cells

Vascular endothelial cells (ECs) constitute the interface between the blood stream and the tissues and in this strategic position ECs appear to perform several key roles in the development of immune and inflammatory responses. In their position between the leucocytes and the extravascular tissue the ECs are in an ideal position to act as immunoregulators of the immune response. ECs can produce amplifying and inhibiting factors which affect the immunoregulation of the immune response (Roska, Geppert & Lipsky, 1984). T-cells at the site of inflammation can produce IFN-γ and induce class II MHC expression on the ECs surface. The T-cells can then recognise antigen in association with self antigen (MHC class II) which results in the EC presenting antigen to the T-cells (Pober *et al.*, 1984; Wagner, Vetto & Burger, 1984). Researchers have developed many *in vivo* models to study the interaction between ECs and leucocytes (reviewed by Harlan *et al.*, 1991) and most of the following information has been gleamed from these studies.

1.9.3 Adhesion of leucocytes

As inflammation develops extravasation of leucocytes to the site of inflammation is seen. Enhanced PMN margination characteristically occurs at sites of acute inflammation,

whereas monocyte interactions with the microvasculature are seen more often in chronic inflammatory reactions (Allison, Smith & Wood, 1955; Wilkinson & Lackie, 1979; Harlan, 1985).

The adhesion of lymphocytes is the first step in their passage from the circulation into the tissue, the site of chronic inflammation. Lymphocytes have been shown to leave the blood and enter lymphoid tissue by binding to the endothelium of specialised postcapillary venules known as high endothelial venules (HEV), followed by migration into the perivascular space (Herman, Yamamoto & Mellins, 1972). HEV-like vessels are present in chronically inflamed gingival tissue and in gingival tissues developing inflammation, but are absent from healthy gingivae (Wynne, Walsh & Seymour, 1988). These HEV-like vessels found in the oral cavity are morphologically similar to lymphoid HEV, suggesting that these HEV may represent sites where the lymphocytes can enter into chronically inflamed lesions. HEV presence in chronically inflamed tissue may indicate local lymph-like tissue presence.

1.9.4 The role of cytokines in cellular migration

Adhesion of leucocytes appears to be essential in controlling cellular traffic into inflamed areas and it has been proposed that cytokines may play an important role in regulation of this traffic. This may be mediated in part by effects of cytokines on EC, both in promoting the

expression of EC adhesion molecules for leucocytes (Pober, 1988; Detmar *et al.*, 1992; Kirnbauer *et al.*, 1992) and in stimulating EC to facilitate leucocyte migration through the vessel wall (Oppenheimer-Marks and Ziff, 1988; Moser *et al.*, 1989).

1.9.5 The cellular adhesion molecules

Leucocyte entry into tissues is controlled by the dynamic interaction between adhesion molecules expressed by leucocytes and the endothelium. Three families of adhesion molecules participate in this vital process, they are;

(1) The *selectins* or the lectin-epidermal growth factor-complement related cell adhesion molecules (LECCAMs) which include the L-selectin (formerly Mel-14, LAM-1 = leucocyte adhesion molecule-1), E-selectin (ELAM-1 = Endothelial leukocyte adhesion molecule 1) and P-selectin (CD62, GMP140);

(2) The *integrins* specifically the lymphocyte function associated antigen-1 (LFA-1) (also known as CD11a/CD18), CR3 (Mac-1, or CD11b/CD18), p150,95 (CD11c/CD18) and very late antigen-4 (VLA-4) (CD49d/CD29);

(3) The *immunoglobulin superfamily* which includes intercellular adhesion molecule-1 (ICAM-1), ICAM-2 (Staunton, Dustin & Springer, 1989) and vascular cell adhesion molecule-1 (VCAM-1).

A vast bank of literature exists on the above adhesion molecules (for review see Hynes, 1987; Stoolman, 1989;

Osborn, 1990; Springer, 1990; Harlan & Liu, 1991). It is however useful to consider the adhesion molecules studied in the proceeding chapters i.e. ELAM-1, ICAM-1, VCAM-1 and LFA-1.

1.9.5.1 ELAM-1

ELAM-1, a member of the selectin family, whose primary sequence consists of a amino terminal lectin-like domain, an epidermal growth factor domain and six tandem repetitive motifs (approximately 60 amino acids each) related to those found in complement regulatory proteins (Bevilacqua *et al.*, 1987, 1989). The ELAM-1 ligand has been discovered to be rich in Lewis X and sialylated Lewis X and found in abundance on the surface of PMNs (Fukuda *et al.*, 1984; Symington, Hedges & Hakomori, 1985; Lowe *et al.*, 1990; Phillips *et al.*, 1990; Walz *et al.*, 1990). ELAM-1 acts as a selective adhesion molecule for PMNs on the surface of cytokine stimulated EC (Bevilacqua *et al.*, 1987, 1989; Picker *et al.*, 1991; Rohde *et al.*, 1992) and is responsible for their migration into tissue. ELAM-1 is also involved in adhesion of skin homing memory T-cells (Rustin *et al.*, 1990; Mackay, 1991; Picker *et al.*, 1991, Shimizu *et al.*, 1991;).

PMN infiltration and ELAM-1 expression has been studied *in vivo* and it has been shown that ELAM-1 expression and PMN infiltration follow similar paths. Both can be seen by 6 hours and peak at 24 hours to a level which is maintained

for 72 hours (Norris *et al.*, 1991). ELAM-1 expression by cultured EC or by endothelium *in situ* in cutaneous explants is maximal 4 to 6 hours after stimulation by IL-1 or tumor necrosis factor (TNF) and spontaneous decline to near baseline expression is seen by 24 hours in the continuous presence of the stimulatory cytokine (Pober *et al.*, 1986; Messadi *et al.*, 1987; Wellicome *et al.*, 1990). EC can become refractory to restimulation by the same cytokine while retaining the ability to respond to another cytokine (Pober *et al.*, 1986) and the use of combinations of cytokines can result in prolonged expression of the adhesion molecule ELAM-1 (Leeuwenberg *et al.*, 1990; Doukas & Pober 1990).

Unstimulated human EC are negative for ELAM-1 (Bevilacqua *et al.*, 1987). Normal human skin is also ELAM-1 negative but ELAM-1 expression can be found restricted to post-capillary HEV (Rohde *et al.*, 1992). ELAM-1 can be induced *in vitro* by cytokines i.e. by IL-1 (Bevilacqua *et al.*, 1985, 1987, 1989; Pober *et al.*, 1986; Groves *et al.*, 1992); TNF (Gamble *et al.*, 1985; Doukas & Pober 1990; Thornhill & Haskard, 1990; Norris *et al.*, 1991) or LPS (Schleimer & Rutledge, 1986; Messadi *et al.*, 1987; Pober *et al.*, 1986) but not by IFN-γ (Pober *et al.*, 1986; Schleimer & Rutledge, 1986; Thornhill & Haskard, 1990).

1.9.5.2 ICAM-1/LFA-1

Immune responsiveness of lymphocytes to antigen and several other effector activities of leucocytes requires cell-cell contact and adhesion (Lipsky & Rosenthal, 1975; Inaba & Steinman, 1984). ICAM-1 is an important adhesion molecule that acts as the receptor for LFA-1 (Rothlein *et al.*, 1986; Lo *et al.*, 1989). ICAM-1/LFA-1 is one of at least three mechanisms involved in lymphocyte adhesion (Dustin & Springer, 1988), with possibly the existence of a second LFA-1 ligand (Rothlein *et al.*, 1986) These two molecules, ICAM-1 and LFA-1, are important adhesion strengthening molecules involved in antigen presentation and other cell-cell interactions involving lymphocytes such as transepithelial trafficking (Dustin *et al.*, 1986; Rothlein *et al.*, 1986).

ICAM-1 is a cell surface protein of molecular weight 90 kDa (Rothlein *et al.*, 1986), is a member of the immunoglobulin superfamily and is found on non-hematopoietic cells (ie vascular endothelial cells, thymic epithelial cells, other epithelial cell and on fibroblasts), as well as on hematopoietic cells (ie tissue macrophages, mitogen stimulated T lymphocyte blasts, antigen presenting cells and germinal centre dendritic cells in tonsils, lymph nodes and peyers patches). Interestingly, LCs have been shown to be ICAM-1 negative (Dustin *et al.*, 1986).

LFA-1, a member of the subgroup of the integrin superfamily the leucocyte integrins, and is the ligand for ICAM-1. The integrins share a common beta subunit (CD18) associated noncovalently with one of three different alpha subunits, designated CD11a (LFA-1), CD11b (Mac-1) and CD11c (p150,95). Of the three integrins, LFA-1 (CD11a/CD18) has been the most extensively studied, and its importance in adhesion is the best understood (Dustin & Springer, 1988, 1989). LFA-1 an αβ heterodimer (180 kDa alpha chain and 95 kDa beta chain) is expressed on the membrane of all lymphocytes (Springer *et al.*, 1982; Hildreth *et al.*, 1983).

ICAM-1 is constitutively expressed on vascular ECs within skin (Majewski *et al.*, 1991); and within lymph nodes and Peyers patches, ICAM-1 expression has been shown to be preferentially located within the T-cell areas (Dustin *et al.*, 1986). During inflammation ICAM-1 expression increases on antigen presenting cells and on ECs, resulting in increased adhesion of T-cells (Lewis *et al.*, 1989; Oppenheimer-Marks, Davis & Lipsky, 1990), PMNs (Barton *et al.*, 1989; Lo *et al.*, 1989) and memory T-cells (Griffiths & Nickoloff, 1989; Buckle & Hogg, 1990) and their subsequent migration into the inflamed tissue. It has also been suggested that ICAM-1 may be involved in the early stages of human thymic development (Singer *et al.*, 1990).

ICAM-1 is constitutively expressed by EC and its expression can be upregulated by IL-1 (Bevilacqua *et al.*, 1985;

Cavender *et al.*, 1986, 1988; Dustin *et al.*, 1986; Haskard *et al.*, 1987; Wellicome *et al.*, 1990; Detmar *et al.*, 1992), TNF (Cavender *et al.*, 1988; Doukas & Pober, 1990; Krutmann *et al.*, 1990), LPS (Schleimer & Rutledge, 1986; Yu *et al.*, 1986; Cavender *et al.*, 1988) and by interferon-γ activated ECs (Yu *et al.*, 1985; Dustin *et al.*, 1986, 1988; Barker, Allen & MacDonald, 1989; Thornhill & Haskard, 1990) and down-regulated by other cytokines i.e. interleukin-4 (IL-4) (Thornhill & Haskard, 1990). ICAM-1 expression on cultured ECs can be increased by the action of TNF, IL-1 and LPS with expression peaking at 7 to 10 hours and still being maintained after 72 hours (Wellicome *et al.*, 1990). Unlike ECs, keratinocytes do not normally express ICAM-1, but expression can be induced *in vitro* and *in vivo* by cytokines (TNF or IFN-γ but not IL-1 or LPS) (Griffiths, Voorhees & Nickoloff, 1989; Barker *et al.*, 1989; Caughman, Lian-Jie & Degitz, 1990; Krutmann *et al.*, 1990; Trefzer *et al.*, 1991) and by ultra-violet-β (Kirnbauer *et al.*, 1992; Krutmann *et al.*, 1992).

1.9.5.3 VCAM-1

When initial studies on EC-leucocyte adhesion began, two cytokine-induced adhesion molecules had been characterised and cloned, ELAM-1 and ICAM-1. Data from a number of laboratories showed that neither ICAM-1 nor ELAM-1 could fully account for the lymphocyte adhesion to cytokine treated human umbilical vein endothelial cells (HUVECs) (Haskard *et al.*, 1986; Dustin & Springer, 1988). Further

more, in patients genetically deficient in CD18, and hence expressing no leucocyte LFA-1, lymphocyte recruitment was essentially normal, despite profound defects in recruitment of phagocytic cells (Anderson & Springer, 1987; Larson & Springer, 1990) indicating the existence of an ICAM-1/LFA-1 independent adhesion mechanism for lymphocytes. Cell lines also existed that bound through neither ELAM-1 nor LFA-1 providing evidence that another adhesion pathway was operating on the cytokine treated HUVECs (Osborn *et al.*, 1989). From this work, Osborn *et al.* (1989) cloned a new adhesion molecule and called it VCAM-1. VCAM-1 is expressed on endothelial cells and binds lymphocytes but not PMNs (Osborn *et al.*, 1989), it consists of six immunoglobulin domains and can be placed in the immunoglobulin superfamily. The ligand for this new adhesion molecule VCAM-1 has been shown to be VLA-4, one of the integrins, with VCAM-1/VLA-4 playing a major role in mononuclear leucocyte migration to sites of inflammation, *in vivo* (Elices *et al.*, 1990).

Following the development of monoclonal antibodies directed against VCAM-1 and studies on cytokine-stimulated endothelium *in vitro* (Osborn *et al.*, 1989). It has been shown that cell surface VCAM-1 is detectable within two hours of cytokine stimulation, is maximal at 10 to 24 hours and is maintained for at least 72 hours in the continued presence of cytokine (Thornhill & Haskard, 1990; Wellicome *et al.*, 1990).

The surface expression of ELAM-1, ICAM-1 and VCAM-1 on HUVECs *in vitro* has been shown to increase after cytokines treatment (IL-1, TNF and LPS), with very little selectivity in the cytokines ability to induce expression of the adhesion molecules. However, recent studies suggest that the cytokine IL-4 shows such selectivity, by increasing adhesion of T-cells but not PMNs to endothelium by inducing an adhesion pathway which is ICAM-1/LFA-1 independent (Thornhill & Haskard, 1990; Thornhill *et al.*, 1991). IL-4 was shown to induce the antigen defined by the monoclonal antibody 1.4C3, later identified as VCAM-1 (Masinovsky, Urdal & Gallatin, 1990).

Immunohistochemical studies have been performed to evaluate the pathophysiologic tissue distribution of VCAM-1 and in general little or no vascular staining of VCAM-1 has been found in normal tissues. In contrast VCAM-1 is expressed at inflammatory sites, with positive vascular tissue seen in inflamed appendix, delayed hypersensitivity reactions and insect bites (Rice & Bevilacqua, 1989; Rice, Munro & Bevilacqua, 1990); within rheumatoid arthritic synovium (Koch *et al.*, 1991) and more recently on vascular tissue of rejecting human cardiac allografts (Briscoe *et al.*, 1991).

1.9.6 Studies on adhesion molecule expression in gingival tissue

Adhesion molecule expression is induced early during experimental inflammatory responses in the skin of monkeys

(Munro, Pober & Cotran, 1989) and humans (Norris *et al.*, 1991). Within gingival tissue very few studies have been carried out, to date. The presence of ICAM-1 on healthy junctional epithelium was reported by Crawford & Hopp, (1990) and a subsequent study (Crawford, 1992) has demonstrated the presence of ICAM-1^{+} diseased pocket epithelium in subjects suffering from adult periodontitis. To date, no studies have examined ELAM-1 or VCAM-1 expression in gingival tissue though a study has examined the presence of other adhesion molecules such as laminin, fibronectin, vitronectin and tenascin within baboons (Steffensen *et al.*, 1992) and CD11b expression on crevicular neutrophils (Watanabe, Hagen & Andersen, 1991).

1.10 Aims

The main aims of this thesis were, firstly, to study the immune and inflammatory cellular infiltrate during the development of gingival inflammation utilising the experimental gingivitis model; secondly, to examine the effect of proinflammatory cytokines on adhesion molecule expression using gingival organ culture and finally, to draw conclusions on the interactions and the roles played by the different variables analysed in this thesis.

In order to study the development of gingival inflammation the experimental gingivitis model of Löe *et al.* (1965) was used. This model has many advantages, it allows gingival biopsies to be taken longitudinally under controlled

experimental conditions; causes minimal discomfort to the volunteers; allows maximal use of the volunteers (i.e. GCF collection as well as gingival biopsies) and the lesions are reversible upon re-institution of oral hygiene (Löe *et al.*, 1965).

To study the development of inflammation within gingival tissue, biopsies were obtained from the subjects participating in the experimental gingivitis studies. The availability of clinically 'healthy' and experimentally inflamed gingival tissue allows the cellular and vascular adhesion molecules changes to be studied in a cross-sectional fashion. It was considered advantageous to obtain diseased tissue from patients attending the Periodontal Unit for routine periodontal treatment, as this would not interfere with normal treatment. The different types of tissue were classified into health, gingivitis and periodontitis.

Immunohistology and computerised image planimetry was employed. Qualitative and quantitative changes in cellular infiltration (PMNs; T-cells and T-cell subsets i.e. helper, suppressor, naive and memory) was assessed as inflammation developed over 21-days and 10-days. Single staining avidin-biotin-complex method utilising peroxidase as the substrate (ABC-P) was used as the immunohistological staining method. The ABC-P method is very sensitive and

gives permanently stained tissue sections which allows enumeration to be carried out at a later date.

The expression of the adhesion molecules (ELAM-1, ICAM-1, VCAM-1 and LFA-1) has been studied in a variety of tissues (Rice & Bevilacqua, 1989; Rice *et al.*, 1990; Majewski *et al.*, 1991; Norris *et al.*, 1991; Rohde *et al.*, 1992). Adhesion molecule expression was studied in clinically 'healthy', experimentally inflamed and diseased gingival tissue. This study was performed in order to determine if the kinetics of adhesion molecules varied within the different types of tissue. Studies were also performed to determine the effect of different cytokines on the intensity of adhesion molecule expression and the effects on the percentage of adhesion molecule positive vessels within cultured explants of gingival hyperplastic tissue. Hyperplastic tissue was used in the organ culture studies and was shown to be immunologically healthy but overgrown. Therefore, large blocks of tissue were available after routine gingivectomy surgery for the gingival organ culture experiments.

Interleukin-1 level changes within GCF was evaluated using a bioassay, to assess if changes in IL-1β could be related to the extent of gingival inflammation. GCF was collected during the 21-day experimental gingivitis study. In addition, changes in LCs numbers as well as the class II MHC positivity of these LCs was assessed within the human

gingival tissues during clinical 'health' and experimental inflammation (21-days and 10-days). This was to determine if alterations within LC numbers are seen as inflammation develops and if their antigen presenting capability varies i.e. MHC positivity.

Chapter 2

Subjects, Materials and Methods

2.1 Introduction

This chapter is in two parts; Part A deals with subject selection, clinical study design, clinical indices and the types of samples collected and Part B describes the materials and techniques used.

Part A: Clinical information

2.2 Subjects

All patients and subjects who participated in the following studies were associated with or were attending the Periodontal Unit of the Glasgow Dental Hospital & School. The experimental protocols were explained in detail prior to the commencement of the studies and copies of the experimental protocols given to the individuals. All individuals were given the option of withdrawing from the studies at anytime. Only one individual (subject F in the 21 day experimental gingivitis study) exercised this option. Ethical approval was obtained from the local ethical committee.

2.2.1 Subjects for the experimental gingivitis studies

Eleven individuals (subjects A-K) consented to participate in these studies, where gingival inflammation was experimentally induced using the experimental gingivitis model of Löe *et al.*, (1965). All subjects were dental students who had volunteered to participate in the studies. All were dentally aware and had no evidence of periodontal

disease. Subjects A - F (age range 22 to 23; 5M:1F) abolished oral hygiene procedures for 21 days and subjects G - K (age range 21 to 22; 5M) for 10 days.

2.2.1.1 Experimental gingivitis study design

All volunteers received oral hygiene instruction and frequent prophylaxis two weeks prior to the commencement of the experimental phase, until clinical gingival health (PI<1, MGI=0) was obtained. All subjects were then instructed to cease all oral hygiene measures and plaque was allowed to accumulate undisturbed for a period of 21 days (subject A - F) or 10 days (subjects G - K). Oral hygiene measures were then re-introduced and volunteers were monitored for a further 14 days until gingival health was re-established.

2.2.1.2 Sample collection: 21 day study

During, before and after the experimental gingivitis phase clinical changes at the mid-buccal aspect (the biopsy site) were evaluated every 7 days for the 21 day study, using the plaque index (PI) of Silness & Löe, (1964) (section 2.3.1) and the modified gingival index (MGI) of Lobene *et al.* (1986) (section 2.3.2).

Gingival crevicular fluid (GCF) samples were taken on day 0, 7, 14 and 21, from the mid-buccal aspect of the first permanent molar to be biopsied (section 2.5.1). GCF samples could not be reliably taken after biopsying a site

and therefore GCF sample numbers varied during the course of the study i.e. on day 0 each individual gave four GCF samples, day 7 three GCF samples, day 14 two GCF samples and on day 21 one GCF sample. Following GCF collection the first permanent molars were biopsied using a pre-determined order (section 2.7.1.1). One volunteer (subject F) declined to give the last two biopsies, but was still sampled for GCF. Thus six biopsies were taken on day 0 and day 7 and five on day 14 and day 21, but only five biopsies were used for the immunohistochemical analysis at each time point. In total 20 biopsies and 24 GCF strips from the biopsy sites were obtained for the 21 day experimental gingivitis study.

2.2.1.3 Sample collection: 10 day study

For the 10 day study the clinical indices PI (section 2.3.1) and MGI (section 2.3.2) were recorded every 3 days at the biopsy site (i.e. mid-buccal aspect) and at the gingival crevicular washing site (GCW) (mesial aspect) of the first permanent molars. Thus on day 0 for each individual, eight clinical scores were recorded (four mid-buccal and four mesial scores), day 3 six scores, day 7 four scores and on day 10 two scores.

Following clinical evaluation GCF strips were collected from the mid-buccal aspect (section 2.5.1) followed by gingival washing from the mesial aspect (section 2.6.2) and finally the gingival biopsies from the mid-buccal aspect

(Fig. 2.1). The gingival biopsies were taken from the first permanent, molars with the remaining first molars being biopsied in a pre-determined order (section 2.7.1.1). In total, 20 gingival biopsies and 20 GCW (from the biopsy sites) were obtained during the 10 day experimental study. GCW were also collected from the unbiopsied sites (n=30).

2.2.2 Subjects for organ culture study

Gingival tissue from three individuals was used for the organ culture studies. All individuals were male Caucasians who suffered from drug-induced gingival hyperplasia, one following epanutin therapy (24 years old) and the others following nifedipine drug therapy (53 and 45 years).

2.2.2.1 Tissue source

All individuals underwent routine periodontal surgery to remove hyperplastic gingival tissue. Informed consent was obtained from the individuals for the gingival tissue (which would be normally discarded) to be used for the organ culture study. Gingival tissue was processed within 30 minutes of collection (as discussed in section 2.7).

2.2.3 Periodontitis subjects

Six individuals were selected to participate in this study. These individuals were all Caucasian, had no history of systemic disease and had not received any antibiotics for the past 3 months. Three of these individuals suffered

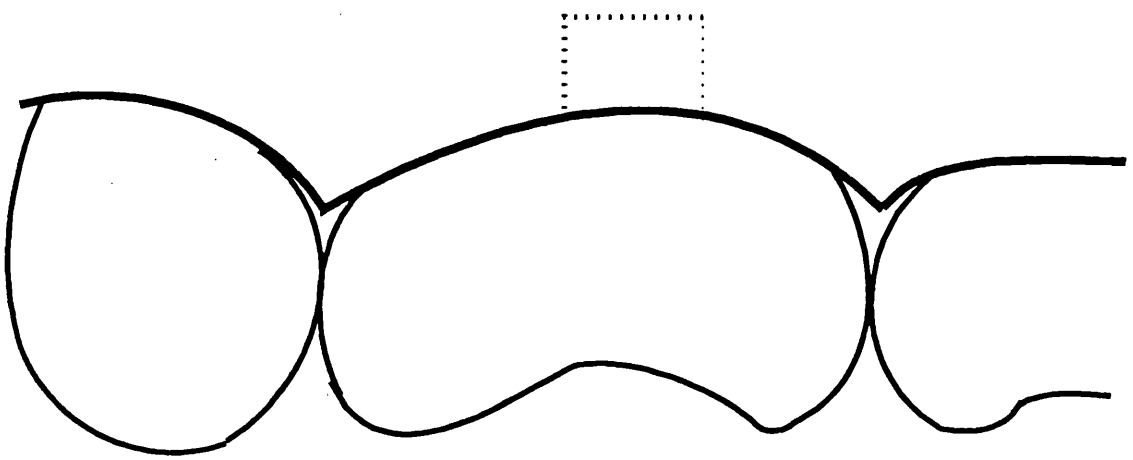

Figure 2.1 Mid-buccal biopsy site.

from adult periodontitis (AP category), two individuals suffered from rapidly progressive periodontitis (RPP category) and one individual suffered from juvenile periodontitis (JP category), as determined by clinical and radiographic examination with JP and RPP subjects diagnosed according to already published criteria (Manson & Lehner, 1974; Page *et al.*, 1983).

Clinical evaluation for the AP, RPP and JP categories (3 AP, 2 RPP, 1 JP) was performed prior to surgery. Gingival inflammation was evaluated using the MGI of Lobene *et al.* (1986) (section 2.3.2) and pocket depth assessed to the nearest millimetre using a Williams periodontal probe (section 2.3.3).

2.2.3.1 Tissue source

During routine periodontal surgery, interproximal diseased tissue which would be normally discarded was taken with the informed consent of the patient. Tissue was immediately embedded and snap frozen, then stored at -70°C until processed (section 2.7).

2.2.4 Subjects for the gingival crevicular washing and lactoferrin study

Twenty-one individuals were selected to participate in this study (age range 20 to 52 years; 7M:14F). The individuals were all Caucasian, had no history of systemic disease and had not received any antibiotics for the past 3 months.

Sixteen of these individuals (APD category) suffered from advanced periodontal disease as determined by clinical and radiographic examination. The remaining five subjects (H category) were classified as periodontally healthy (no attachment loss, pocket depths less than 3mm and no significant gingival inflammation).

2.2.4.1 Sample collection

Sixty-three GCWs were collected using a modification of the Skapski & Lehner method, (1976) (section 2.6.2). A maximum of four sites were sampled per subject. Gingival inflammation of the sites sampled was evaluated using the MGI (section 2.3.2) and the pocket depth assessed (after GCW sampling) to the nearest millimetre using a Williams periodontal probe (section 2.3.3). Diseased sites (MGI≥2, PD≥3mm) were obtained from the periodontitis patients whereas healthy sites (MGI≤1, PD≤2mm) were obtained mainly from the periodontally healthy subjects and in some cases from the periodontitis patients. All GCW samples were processed within 30 minutes of collection (section 2.6.1.4).

2.2.5 Order of sample collection

An order of collection was followed: clinical indices (PI then MGI) were evaluated initially; GCF was then collected; followed by GCWs; and finally the gingival biopsy. This sequence was aimed at minimising the disruption caused by each sampling procedure.

2.3 Clinical Indices

Clinical indices (PI: Silness & Löe, 1964 and the MGI: Lobene *et al.*, 1986) were used to record changes in plaque accumulation and gingival involvement.

2.3.1 Plaque Index

The plaque index (PI) of Silness & Löe (1964) was used to score plaque accumulation in each of the areas of the tooth used for sampling. Each area was given a score of 0 - 3 (Table 2.1) and this was the PI for the area.

2.3.2 Modified gingival index

The modified gingival index (MGI) of Lobene *et al.* (1986) uses a five point scale (Table 2.2) and permits a non-invasive evaluation of the early visible changes in gingival tissue and allows untraumatised gingival biopsies to be taken. The MGI was recorded just after PI scoring to give site scores.

2.3.3 Pocket depth assessment

The probing depth (PD) i.e. the distance from the gingival margin to the bottom of the gingival pocket was measured using a graduated Williams periodontal probe. Pocket depth was assessed to the nearest millimetre.

2.3.4 Sites scored

2.3.4.1 Biopsy, GCF sites and GCW sites

During the experimental gingivitis studies the clinical

Table 2.1 Plaque Index of Silness & Löe, (1963).

0	No film.
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth.
2	Moderate accumulation of soft deposits within the gingival pockets, or on the tooth and gingival margin which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

Table 2.2 Modified gingival index scoring system of Lobene *et al.* (1986).

0	Absence of inflammation.
1	Mild inflammation, slight change in colour, little change in texture of any part of but not the entire marginal or papillary gingival unit.
2	Mild inflammation; criteria as above but involving the marginal or papillary gingival unit.
3	Moderate inflammation, glazing, redness, oedema and/or hypertrophy of the marginal or papillary gingival unit.
4	Severe inflammation, marked redness, oedema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

indices were recorded at each GCW site (mesial aspect), GCF site and biopsy site (mid-buccal aspect) prior to sampling. Both indices were collected every 7 days of the 21-day experimental phase and every 3 days of the 10-day experimental phase, in order to monitor clinical development of gingival inflammation.

2.3.4.2 Patient biopsy sites

PD was used to designate sites 'diseased sites' if MGI≥2, PD≥3mm. PD was assessed using the Williams periodontal probe (section 2.3.3) and 4 sites per biopsy (interdental col) were assessed (mesial-buccal, mesial-lingual, distal-buccal and distal-lingual). The biopsy scores for the four areas were added and divided by four to give the average PD score for each site.

2.3.5 Intra-examiner variability

Since both indices were non-invasive, intra-examiner calibrations were carried out. The need to assess inter-examiner variability was avoided as only one examiner was used during any one trial.

2.3.6 Statistical analysis

2.3.6.1 Association between clinical indices and other parameters

Clinical index scores were generally skewed in their distribution. Therefore to demonstrate association between

the clinical indices (PI, MGI and PD) and other parameters the non-parametric Spearman's rank correlation coefficient was used. Data were analysed using the 'Minitab' statistical package on an IBM PC.

2.3.6.2 Repeated measures of clinical indices

Clinical indices were collected from each subject over four sampling time points i.e. repeated measures. Therefore to demonstrate if days of oral hygiene abstention had an effect on clinical indices the non-parametric Friedman's test (Minitab) was used.

Part B: (I) Materials

2.4 Materials: Buffers and Reagents

All chemicals were analytical grade, obtained from BDH Chemicals Ltd., Poole, Dorset (UK) unless otherwise stated.

2.4.1 General use

2.4.1.1 Phosphate buffered saline (PBS)

PBS pH 7.4, 1.5mM KH_2PO_4, 10mM $Na_2HPO_4.H_2O$, 0.14M NaCl and 2.7mM KCl was dissolved in 500ml of distilled water, pH was adjusted to 7.4 (using 1M NaOH and 1M HCl) and the final volume adjusted to 1 litre.

2.4.2 Materials used during GCF sampling

2.4.2.1 Filter paper strips

Whatman grade 4 filter paper was obtained from Whatman International Ltd (UK). Using a steel ruler and a scalpel, 2x13mm strips were cut manually, as recommended by previous investigators (Griffiths, Curtis & Wilton, 1988) and sterilized by autoclaving. Sterilized strips were stored at room temperature in glass universals.

2.4.2.2 GCF elution buffer

GCF elution buffer was prepared using RPMI 1640 medium (Gibco, UK) supplemented with 5% foetal calf serum (Globepharm, UK), 30μg/ml gentamicin (Gibco, UK) and 2.5μg/ml Amphotericin B (Gibco, UK).

2.4.2.3 Recombinant human Interleukin-1β

Recombinant human interleukin-1β was a gift from Dr A. Shaw, Glaxo, Geneva, Switzerland.

2.4.2.4 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (DDT)

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, analytical grade (SIGMA, UK).

2.4.2.5 Anti-Interleukin-1β

Anti-IL-1β sheep polyclonal serum (S77β) was a gift from Dr A. Shaw, Glaxo, Geneva, Switzerland.

2.4.3 Materials used during crevicular PMN isolation and processing

2.4.3.1 Leishmans stain

Leishmans stain (SIGMA, UK) was prepared as 0.2% w/v in methanol i.e. 0.2g dissolved in 100ml methanol. The stain was filtered before use.

2.4.3.2 Coating buffer

Carbonate-bicarbonate buffer prepared by dissolving 1.5g Na_2CO_3, 2.93g $NaHCO_3$ and 0.2g NaN_3 in 950ml of distilled water with the final volume adjusted to 1 litre. The pH was adjusted to 9.6 using 1M HCl.

2.4.3.3 Incubation buffer (PBS-Tween 20, 0.1% BSA)

Incubation buffer (PBS-Tween 20, 0.1% BSA) solution was prepared by adding 0.5g of Tween 20 (0.05%) and 1g lyophylised bovine serum albumin (BSA) (0.1%) to 900ml of PBS, pH 7.4 (section 2.4.1.1) and the final volume adjusted to 1 litre.

2.4.3.4 Wash buffer (PBST)

A stock solution of 10 times concentrated incubation buffer (nil BSA) was prepared. This was diluted 1 in 10 prior to use i.e. 1 part PBST: 9 parts distilled water.

2.4.3.5 Substrate buffer

The substrate buffer was prepared by dissolving 0.95g

citric acid and 1.4g $Na_2HPO_4.H_2O$ in 100ml of distilled water (pH 5). The substrate buffer was prepared fresh just before use by adding 40mg ortho-phenylene diamine (OPD) (SIGMA) and 40µl H_2O_2.

2.4.3.6 Purified lactoferrin standard and antisera for ELISA

Purified lactoferrin (LF) (5mg/ml) was purchased from Calbiochem (Novabiochem Ltd., Nottingham, UK). The LF standard came lyophilised and was reconstituted according to the manufacturer's instructions. Goat anti-LF and rabbit anti-LF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire, UK). The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA) and were fractionated. All reagents, after reconstitution (if required), were aliquoted and stored at -20°C until used. Reagents were replaced regularly to avoid deterioration.

2.4.4 Materials used during biopsy tissue processing

2.4.4.1 Embedding medium

Optimal cutting temperature compound (O.C.T.) (Tissue-Tek II) was obtained from Shandon Southern Products Ltd, Cheshire, UK.

2.4.4.2 Vectabond reagent

Vectabond reagent was obtained from Vector Laboratories, Peterborough, UK. Prior to use, the entire contents of the

bottle (7ml) was added to 350ml acetone to make the working solution.

2.4.4.3 Acetone

Acetone technical (Propanone) was obtained from Avondale Lab. Ltd, Oxon, UK.

2.4.4.4 Blocking serum

Horse serum was obtained from Vector Laboratories (Peterborough, UK), as part of an ABC kit. Blocking serum was prepared by adding 3 drops of this horse serum to 10ml PBS pH 7.4, to obtain the working solution.

2.4.4.5 Biotin-conjugated secondary antibodies

Biotin-conjugated horse anti-mouse IgG was obtained from Vector Laboratories (Peterborough, UK), as part of the proprietary ABC kits (ABC-peroxidase and ABC-alkaline phosphatase). Working solutions were prepared prior to use by adding 1 drop of biotin-conjugated secondary antibody to 10ml PBS pH 7.4.

2.4.4.6 Deperoxidising solution

Deperoxidising solution was prepared by adding 1ml of 30% H_2O_2 to 99ml methanol to give 0.03% methanolic hydrogen peroxide.

2.4.4.7 ABC-peroxidase (ABC-P) 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 10ml PBS (pH 7.4) and mixed, this was followed by 2 drops of reagent B, the solution was again mixed. The complex was prepared 30 mins before use and was stored at 4°C.

2.4.4.8 Peroxidase substrate - DAB

3, 3' Diaminobenzidine tetrahydrochloride (DAB) tablets (10mg) were obtained from SIGMA, UK. A stock solution was prepared of 2.5mg/5ml and 30 minutes prior to use 5µl hydrogen peroxide (H_2O_2) was added to give the working solution.

2.4.4.9 Peroxidase substrate - DAB/nickel chloride

An 8% nickel chloride solution was prepared by dissolving 0.8g in 10ml distilled water. The DAB substrate was prepared as outlined in section 2.4.4.8, to this working solution 25µl of the 8% nickel chloride solution was added to give a 4% $NiCl_2$/DAB substrate working solution.

2.4.4.10 100mM TRIS-HCl buffer

A 0.2M solution was prepared by dissolving 24.23g of tris (hydroxymethyl) methylamine [2-amino-2-(hydroxymethyl) propane-1,3-diol) (TRIS)] in 900 ml distilled water,

adjusted to a final volume of 1 litre. TRIS-HCl buffer, pH 8.23 was prepared with 25ml 0.2M TRIS and 22.5ml 0.1N HCl made up to a final volume of 200ml with distilled water.

2.4.4.11 Levamisole (0.1mM)

Levamisole solution was prepared by dissolving 0.024g in 900ml of 100mM TRIS-HCL, pH 8.2 (section 2.4.4.10), adjusted to a final volume of 1 litre to give a 0.1mM levamisole solution in 100mM TRIS-HCl.

2.4.4.12 ABC-alkaline phosphatase (ABC-AP) complex

ABC-AP reagents were obtained from Vector Laboratories (Peterborough, UK) as part of an ABC kit. The solutions were in two bottles reagent A (Avidin DH) and reagent B (biotinylated alkaline phosphatase H). Two drops of reagent A were added to 10ml PBS (pH 7.4) and mixed, this was followed by 2 drops of reagent B, the solution was again mixed. The complex was prepared 30 mins before use and was stored at 4°C.

2.4.4.13 Alkaline phosphatase substrate

Alkaline phosphatase substrate kit I (Vector Red$^{(R)}$) was obtained from Vector Laboratories, Peterborough, UK. Substrate was prepared immediately before use by adding 2 drops of reagent 1 (ABC-AP substrate reagent 1) to 5ml of buffer (100mM TRIS-HCL, pH 8.2) and mixing well, followed by 2 drops of reagent 2 (ABC-AP substrate reagent 2) mixing well and finally 2 drops of reagent 3 (ABC-AP substrate

reagent 3) and mixing well.

2.4.4.14 Potassium iodide (0.06M)

A 0.06M potassium iodide solution was prepared by dissolving 9.96g of potassium iodide in 900ml of distilled water and the final volume was adjusted to 1 litre.

2.4.4.15 Copper sulphate solution

A 0.5% solution was prepared by dissolving 0.5g of anhydrous $CuSO_4$ in 100ml distilled water.

2.4.4.16 Mayer's haematoxylin

Haematoxylin (1g) was dissolved in 1L distilled water, 50g of aluminum alum ($AlNH_4(SO_4)_2.12H_2O$) 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 shaken to dissolve. The 0.1% Mayer's haematoxylin solution was filtered before use.

2.4.4.17 Gurrs Eosin

A 0.5% solution of $CaCl_2$ (0.5g/100ml) was prepared in distilled water and 1g eosin powder was added to make a 1% eosin solution (1g/100ml).

2.4.5 Materials used during organ culture study

2.4.5.1 Hanks buffered salt solution (HBSS)

HBSS (Gibco, UK) was supplemented with 50IU/50mg

Penicillin/streptomycin per ml of medium (Gibco, UK).

2.4.5.2 Medium 199

Medium 199 (Gibco, UK) was supplemented with 10% foetal calf serum (Globepharm, UK), 2mM Glutamine (Gibco, UK) and 50IU/50mg Penicillin/Streptomycin (Gibco, UK) per ml.

2.4.5.3 Control medium (0.1% BSA)

A 1% bovine serum albumin (BSA) solution was prepared by layering 0.2g BSA (SIGMA, UK) onto 15ml of PBS. The solution was allowed to stand at room temperature for 30 minutes, mixed with a magnetic stirrer until dissolved and the final volume adjusted to 20ml. To prepare the control medium a 1:9 dilution was made (1 part 1% BSA: 9 parts Medium 199) (Section 2.4.5.2) this was the working control medium (0.1% BSA).

2.4.5.4 Recombinant Tumour necrosis factorα

Recombinant tumour necrosis factorα was a gift from Dr A. Shaw, Glaxo, Geneva, Switzerland.

2.4.5.5 Lipopolysaccharide

10mg of lyophilised lipopolysaccharide (*Escherichia coli*) was obtained from SIGMA (UK). Reconstituted with PBS (1% BSA) and aliquoted into 10µl aliquots (10µg per 10µl aliquot) and stored at -70°C. The working solution was prepared by making a 1:999 dilution i.e. 1 part LPS to 999 parts of medium 199. Thus the working solution contained

1μg of LPS per ml.

2.4.5.6 Interferon-γ

100μg of recombinant human interferon-γ with $2x10^6$ IU per vial was obtained from Biogen (Switzerland). Reconstituted with PBS (1% BSA) and aliquoted into 5μl aliquots (10,000 IU per aliquot)and stored at -70°C. Before use 0.995ml PBS (1% BSA) was added to each aliquot producing a 1ml solution containing 10,000 IU. The working solution was then prepared by making a 1:39 dilution i.e. 1 part of the 10,000 IU/ml solution to 39 parts of medium 199. Thus the working solution contained 250 IU of recombinant human interferon-γ per ml.

Part B: (II) Methods

2.5 Gingival crevicular fluid

2.5.1 GCF sampling

2.5.1.1 Collection method

The individual crevicular sites were gently air-dried and any visible supragingival plaque removed. The area was carefully isolated with cotton wool rolls and saliva ejector to avoid saliva contamination of the samples. The sterilised filter paper strip (section 2.4.2.1) was introduced into the crevice until mild resistance was felt and left for 30 seconds (Fig. 2.2). Each site was sampled for 30s and only once. Care was also taken to avoid mechanical injury of the tissue.

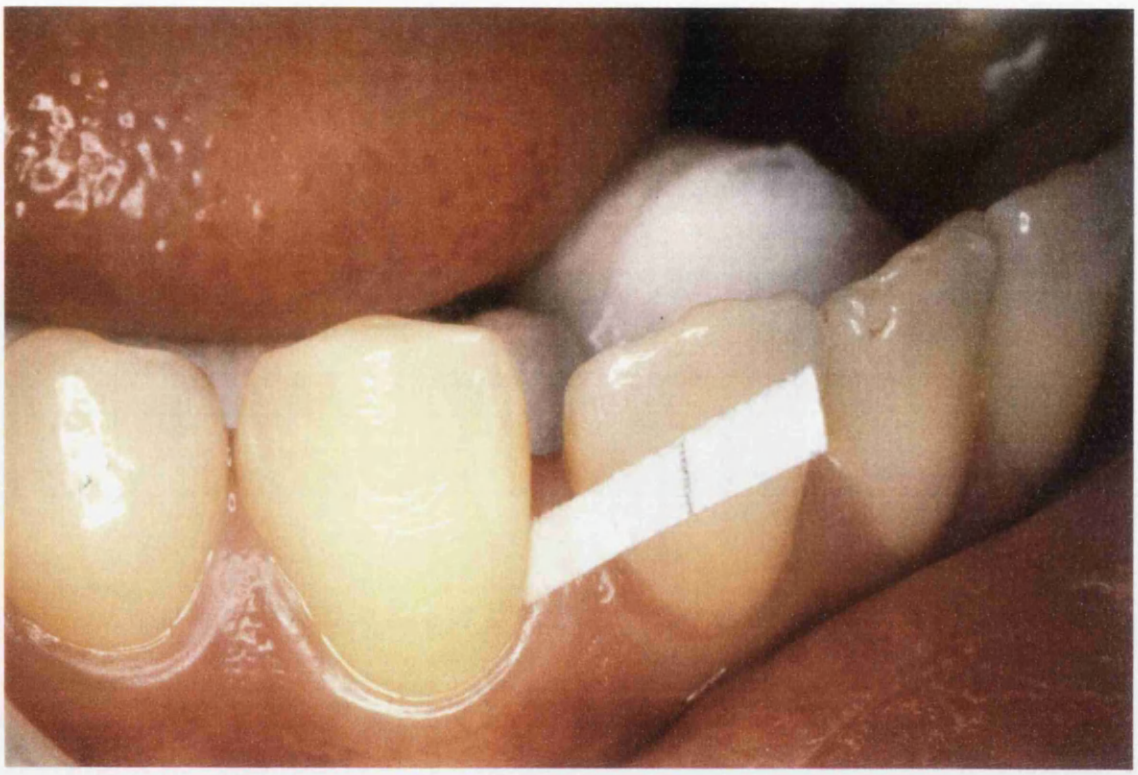

Figure 2.2 Gingival crevicular fluid collection.

2.5.1.2 Quantitation of GCF volume

After GCF collection, the paper strips were transferred to the calibrated chairside-located Periotron (Periotron 6000, Harco Electronics, Winnipeg, Canada) (section 2.5.2) for the quantification of the fluid volume. The jaws of the Periotron were wiped with pure methanol between sequential readings to prevent cross contamination between strips. After collection the strips were placed in individual sterile microcentrifuge tubes and stored at -30°C until elution.

2.5.1.3 Elution of GCF

GCF was eluted from the paper strips by incubation for 30 minutes at 4°C in 150µl of elution buffer RPMI 1640 medium (section 2.4.2.2). After elution, the paper strip was removed and the eluate was centrifuged for 2 minutes in a microcentrifuge to remove any particulate matter. The eluate supernates were then stored frozen at -20°C before being assayed for IL-1.

2.5.1.4 Interleukin-1ß Bioassay

Interleukin-1 activity was bioassayed using the IL-1 sensitive cell line D10(N4)M (Helle, Boeije & Aarden, 1988; Hopkins & Humphreys, 1989) with the modification that the cell proliferation was determined by a colourimetric method utilizing 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Mosmann, 1983). The potency of the stimulatory activity in the eluate was determined from

dose-response curves obtained with a recombinant human DNA-derived IL-1 beta (rhIL-1β) standard using the computer programme ALLFIT (De Lean, Munson & Rodbard, 1978). Addition of sufficient anti-IL-1β serum (a sheep polyclonal serum (S77β): gift of Dr. A. Shaw, Glaxo, Geneva) to completely neutralise the activity of 100 pg of rhil-1β, inhibited at least 70% of the stimulatory activity. This bioassay is particularly sensitive and specific for IL-1 and thus the remaining activity was presumed to be associated with IL-1α.

2.5.1.5 Statistical analysis

Changes in IL-1ß concentration (ng/µl) over time were analysed using the repeated measures analysis of variance test - MANOVA (SPSS), and if significant were further analysed using paired T-tests.

Associations between the clinical parameters and IL-1ß were analysed using Pearson's correlation coefficient and Regression analysis (Minitab). Prior to analysis the mean IL-1ß levels, mid-PI and mid-MGI scores for each subject was summed over the four sampling intervals (Matthews *et al.*, 1990).

2.5.2 Calibration of the Periotron 6000

Prior to commencement of this study a calibration graph was constructed for the Periotron 6000 in order to transform the Periotron digital indications for each filter strip

into volumes. Known volumes of serum diluted 1:1 in PBS were delivered to Whatman grade 4 paper strips with a Hamilton microsyringe in volumes ranging from 0.2 to 1μl, in 0.2μl increments. Each measurement was performed 6 times and the mean values for each volume were used in a regression analysis (Fig. 2.3). The slope, intercept and correlation coefficient of the regression analysis were used to determine the volumes of GCF collected.

Calibration of the Periotron 6000 was carried out daily, prior to GCF sampling to reduce error produced by atmospheric humidity and strip variability. A dry strip was placed between the Periotron jaws after each test strip and when the Periotron 6000 was not in use, a dry strip was placed between the jaws during storage.

2.6 PMN isolation from the crevice

2.6.1 Preliminary study: Method for isolating crevicular leucocytes

2.6.1.1 Introduction

Gingival crevicular leucocytes can be collected by various techniques for quantitation and *in vitro* observation. We tested 3 methods to determine the optimal recovery of crevicular leucocytes for subsequent experiments.

2.6.1.2 GCF and GCW collection

In order to compare GCF and GCW methods of PMN recovery we set up the following experiments.

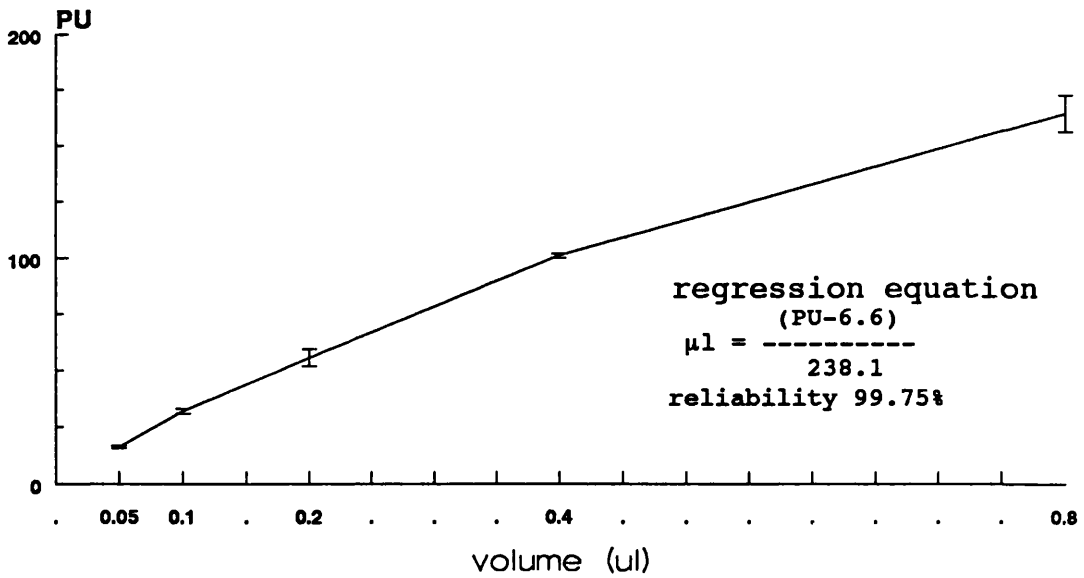

Figure 2.3 Periotron 6000 calibration curve. Periotron units (PU) plotted against volume (µl). Mean and standard deviation of six readings. **Inset** Example of typical regression equation and percentage reliability.

GCF samples were collected from 13 healthy sites. Nine sites were sampled utilising the method employed for GCF collection described previously (section 2.5.1) and the remaining four healthy sites were sampled using a modification of the Skapski & Lehner, (1976) crevicular washing method (section 2.6.2). Briefly the teeth were isolated by using a salivary ejector and cotton wool rolls in the buccal sulcus and any visible supragingival plaque was removed. Aliquots of 10µl of PBS were ejected and reaspirated 6 times using the 100µl Hamilton microsyringe (SGE, Australia) resting interdentally on the surface of the tooth. The gingival washings were collected from sites and stored in microcentrifuge tubes.

2.6.1.3 GCF processing

After GCF collection the filter strips were divided into 2 groups; Group 1 were processed according to the method of Cimasoni & Giannopoulou, (1988) (method A) and Group 2 processed according to the method of Ebersole *et al.* (1984) (method B).

Method A - filter strips were placed in microcentrifuge tubes containing 150µl PBS-Tween (section 2.4.3.3) and vortexed for 30s. Method B - filter strips were placed in a microcentrifuge tube which had a cap placed within it, the cap had a hole in the center to allow fluid to pass through. To this 50µl of PBS-Tween was added and the tubes centrifuged at 3000g for 2 minutes. This was repeated twice giving a total of 150µl passed through. Aliquots

(50µl) of the samples (method A and B) were then cytocentrifuged at 70g (Shandon Scientific Ltd, UK) and the cytospin preparation stained using the Leishman method (section 2.6.3). A differential count was then performed at x400 magnification.

2.6.1.4 GCW processing

GCW were processed using Method C: Within 30 minutes of GCW collection a total cell count was performed on 5µl of the sample. The remainder of the sample was used to perform a differential count of 200-300 cells into polymorphonuclear and mononuclear cells after cytocentrifuging the samples at 70g (Shandon Scientific Limited, UK) and staining the cells by the Leishman method (section 2.6.3). The numbers of PMNs/µl of GCW was finally calculated using the formula: total cell count x % PMNs/100.

2.6.1.5 Choice of technique following preliminary experiments

Using the filter paper methods (A or B), no PMNs could be isolated, whereas with the GCW method (method C), crevicular leucocytes could be recovered from healthy sites.

2.6.2 Modified gingival crevicular washing method

Following the preliminary PMN isolation technique experiments, a modification of the semiquantitative Skapski & Lehner, (1976) technique was used to study crevicular

leucocytes. Briefly GCW were collected after careful isolation of the site. Aliquots of 20µl of PBS were ejected and re-aspirated 3 times using a 20µl micropipette fitted with a flat ended pipette tip (Labsystems, UK) (Fig. 2.4). Washings were then stored in sterile microcentrifuge tubes at 4°C before processing (section 2.6.1.4).

Two major modifications of the Skapski and Lehner technique were:

(1) it was found that re-aspirating 3 times allowed better recovery of the initial GCW volume than re-aspirating 6 times (as recommended by Skapski & Lehner, 1976).

(2) aliquots of 20µl of PBS ejected and reaspirated 3 times using a 20µl micropipette fitted with a flat ended micropipette tip (Labsystems, UK) gave better recovery of the washing than the use of a Hamilton microsyringe.

2.6.3 Leishmans method

Cytospin preparations of GCW were air dried thoroughly before neat Leishmans (section 2.4.3.1) was added. After three minutes the stain was diluted 1:1 with distilled water and left for a further 10 minutes. The preparations were then washed and mounted under Uvinert mountant aqueous solution (BDH, UK).

2.6.4 Lactoferrin quantitation

LF quantitation was performed on the eluate of the GCW. The LF ELISA is based on the technique described by

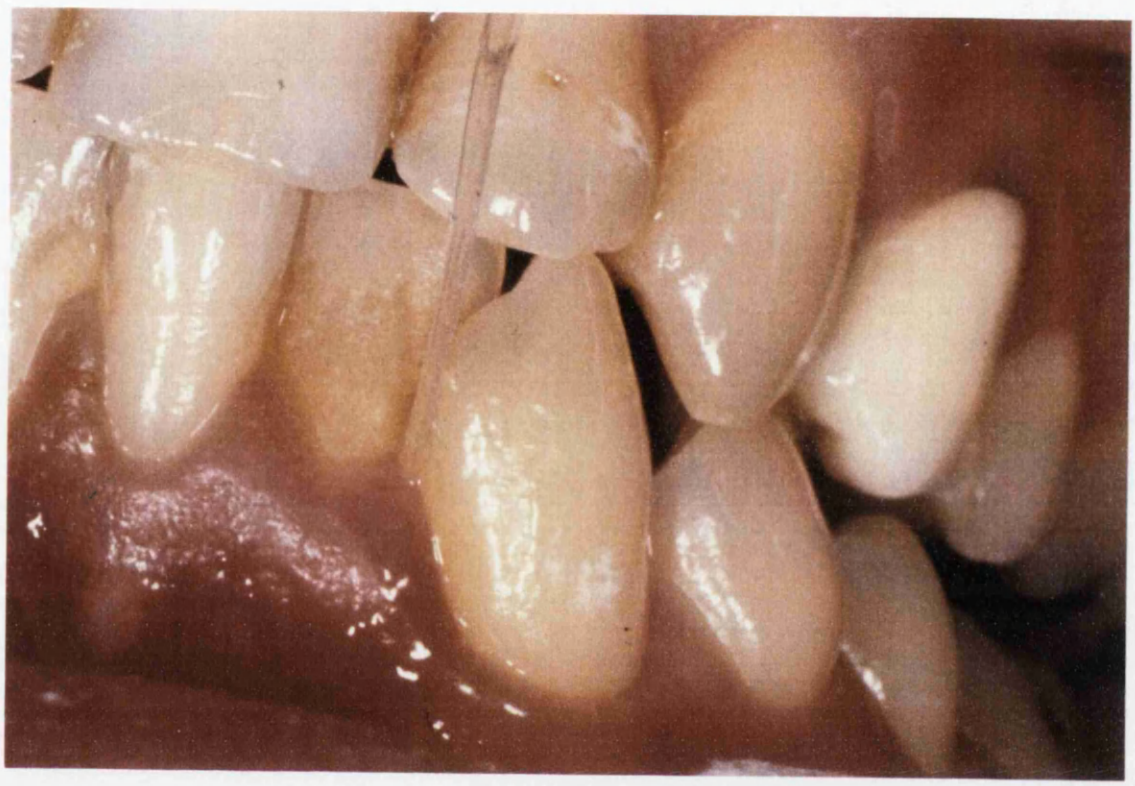

Figure 2.4 Gingival crevicular washing collection.

Hetherington, Spitznagel & Quie, (1983). In summary (Fig. 2.5), the 96-well polystyrene microplate (Immulon IV, Dynatech Laboratories, Sussex, UK) was coated with the first antibody (goat anti-LF). The eluate of the sample was added and any LF present was captured by the immobilised antibody. Incubation with the second specific antiserum developed in rabbit (rabbit anti-LF) was followed by the addition of the HRP conjugated anti-rabbit IgG (goat). Visualisation was achieved by incubation with the substrate (OPD) and stopping the reaction with H_2SO_4. The plate was read at 490nm, and optical densities obtained using the Dynatech Minireader II. In the sandwich assays, the amount of antigen present in the sample is directly proportional to the amount of the second antiserum (rabbit), which was quantified indirectly by the addition of the HRP conjugated anti-rabbit IgG, resulting in high optical densities for high antigen concentrations. The method is described in detail in Table 2.3. Plates included 10 serial two-fold dilutions of purified antigen for the construction of a standard curve (Fig. 2.6). Only the central wells were used when running standards or samples (in triplicate) in an effort to avoid the edge effect. The peripheral wells were used assaying the controls which are shown in Table 2.4. All controls were run in duplicate except for the zero-antigen (control 14; Table 2.4) which was run in quadruplicate. LF levels in GCW samples were expressed as ng/µl GCW.

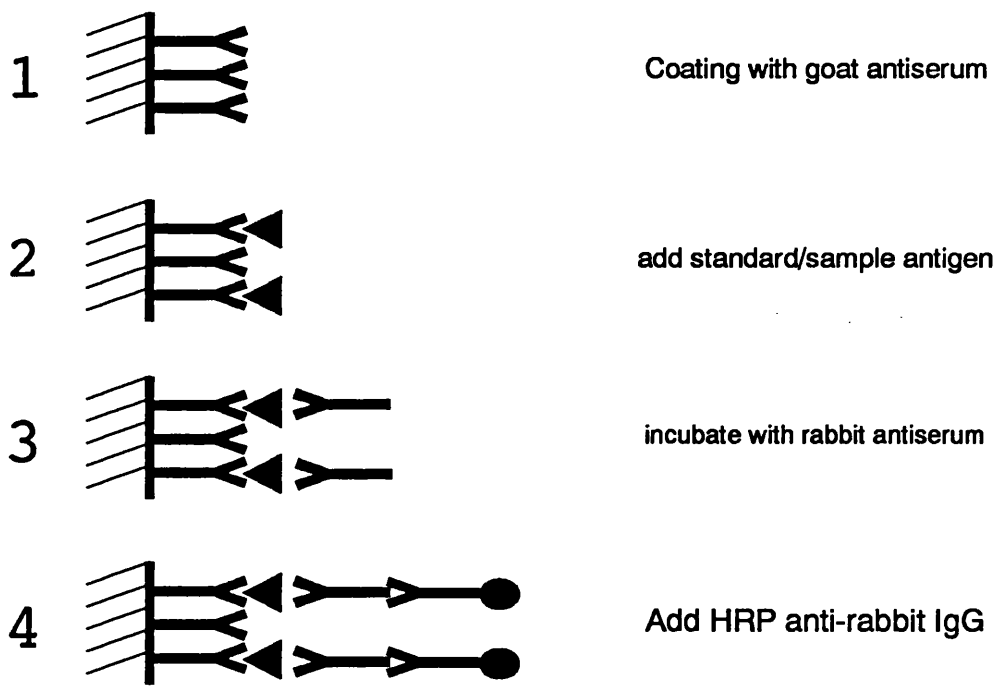

Figure 2.5 ELISA methodology.

Table 2.3 Steps and reagents for the lactoferrin (LF) sandwich ELISA. The LF standards concentration range was 0.24 to 125 ng/ml.

Sandwich ELISA	
Steps and Reagents	
1.	Wash with 200µl/well CB thrice and dry.
2.	Coat with 100µl/well LF antiserum (1:6000 in CB). Incubate overnight at 4°C.
3.	Wash with 200µl/well PBST, 3x6 times, and dry. Removal of excess and loosely coated antibody.
4.	Add 100µl/well standard LF in IB or eluted sample. Incubate 2h at 37°C.
5.	Wash with 200µl/well PBST, 3x6 times, and dry. Removal of free and loosely bound antigen.
6.	Incubate with 100µl/well rabbit antiserum to LF (1:4000 in IB), overnight at 4°C.
7.	Wash with 200µl/well PBST, 3x6 times, and dry. Removal of free and loosely bound antigen.
8.	Add 100µl/well HRP anti-rabbit IgG (Goat) (1:4000 in IB). Incubate for 90 min at 37°C.
9.	Wash with 200µl/well PBST, 3x6 times, and dry. Removal of excess HRP-anti-IgG.
10.	Add 100µl/well SB Incubate for approx. 20 min and stop colour development with 50µl/well 1M H_2SO_4. Read at 490nm.

CB: coating buffer; HRP: horseradish peroxidase; IB: incubation buffer; LF: lactoferrin; PBST: phosphate buffer saline - tween

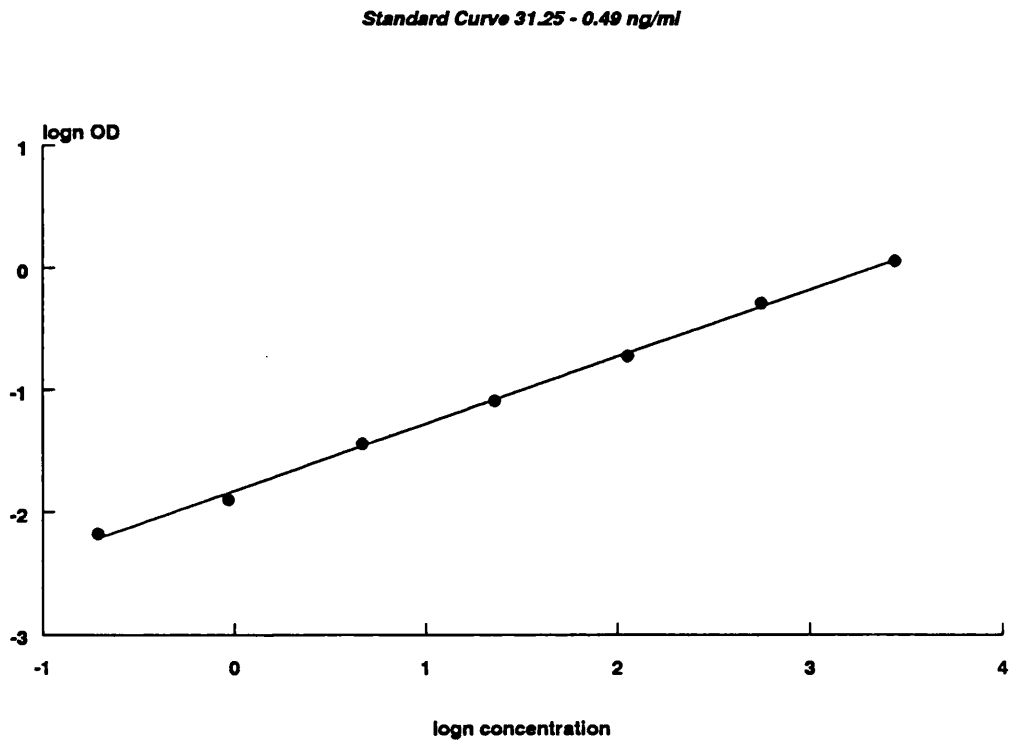

Figure 2.6 Lactoferrin standard calibration curve.

Table 2.4 Control tests for the lactoferrin sandwich ELISA. When the addition of a reagent was omitted the equivalent volume of the respective buffer alone was added. Coating refers to the goat antiserum. Standard was added at 2^{-5} dilution of its starting concentration. The antiserum refers to the second antibody (rabbit).

Control	Step			
	Coating	Standard	Antiserum	HRP conjugate
1.	-	-	-	-
2.	+	-	-	-
3.	-	+	-	-
4.	-	-	+	-
5.	-	-	-	+
6.	+	+	-	-
7.	+	-	+	-
8.	+	-	-	+
9.	-	+	+	-
10.	-	+	-	+
11.	-	-	+	+
12.	+	+	+	-
13.	+	+	-	+
14.	+	-	+	+
15.	-	+	+	+

2.6.5 Statistical analysis

The distribution of LF levels and PMN numbers in the GCW were skewed and were logarithmically transformed to the base 10 (log^{10}) to normalise their distribution for further analysis. Association between LF levels (ng/µl GCW) and PMN numbers (PMN/µl GCW) was determined by the Pearson's correlation coefficient (section 2.7.7.3) on the log^{10} transformed data. Data was analysed using the 'Minitab' statistical package on an IBM PC.

2.7 Gingival biopsies

2.7.1 Biopsy sites

2.7.1.1 Experimental gingivitis

Gingival biopsies (2mm x 2mm) were obtained during the experimental gingivitis studies. They were taken every three days during the 10 day study and every seven days during the 21 day study. The biopsies were taken using two vertical incisions joined by a horizontal incision from the mid-buccal aspect (Fig. 2.1) of the lower right first permanent molar on day 0 and the remaining first permanent molars were then biopsied in rotation (day 3 (day 7) - upper right, day 7 (day 14) - upper left and day 10 (day 21) - lower left). All the biopsy sites were covered in Coepack dressing and healed uneventfully, in total four biopsies were taken from each individual. Twenty gingival biopsies were collected for the 21 day study and 20 for the 10 day study.

2.7.1.2 Patient tissue

For the studies involving periodontitis patients (section 2.2.3) the interproximal tissue was used and in total 19 sites were sampled. For the organ culture study the tissue used was removed from patients (section 2.2.2) who were undergoing a standard gingivectomy procedure to remove hyperplastic gingival tissue.

2.7.2 Sectioning and storage of gingival tissue

All tissue biopsies (experimental gingivitis, patient and organ culture) were embedded in Tissue Tek O.C.T. and snap frozen to prevent ice crystal formation. Eight micrometer thick serial section were then cut using a Microcryostat (Shandon Scientific, UK) and sections were collected on vectabond treated slides (section 2.4.4.2) and stored at -70°C until further processing.

2.7.3 Pre-treatment of glass slides

During processing of frozen tissue sections for immunohistochemical staining, sections commonly become detached from the glass slide. Vectabond reagent (Vector Laboratories, Peterborough, UK) chemically modifies the glass to form a highly adherent surface.

Vectabond pre-treatment of glass slides prevents frozen tissue from detaching and decreases background staining. Briefly, untreated glass slides were rinsed in acetone (BDH, UK) and placed in Vectabond reagent solution (section

2.4.4.2) for 5 minutes, followed by a brief rinse in distilled water. Treated slides were then dried overnight at 37°C before being stored at room temperature.

2.7.4 ABC Techniques

2.7.4.1 Preliminary studies to determine optimal staining method

Numerous combinations of reagent were employed in an attempt to determine the optimal staining method for single staining (ABC-P or ABC-AP) and double staining (ABC-P followed by ABC-AP) as outlined in Table 2.5 and 2.6.

2.7.4.2 Single staining comparisons

The results from the single staining preliminary studies indicated (Table 2.5):

(1) Blocking before the addition of the primary monoclonal antibody (mAb) gave better staining with both detection systems (ABC-P and ABC-AP) i.e. less background (combinations 1 and 2 and 5 and 6);

(2) Deperoxidation to remove endogenous peroxidase if performed before the primary monoclonal antibody was labelled with biotin conjugated antibodies resulted in altered primary mAb such that staining was reduced (combination 3). Staining was optimal if deperoxidation was carried out after biotin labelling (combination 4);

(4) ABC-AP substrate Vector red gave better staining if levamisole (0.1mM) was included in the solution (combination 7 and 8);

Table 2.5 Combinations of single staining used during preliminary studies in order to determine optimal staining procedure.

	Single staining procedures combinations	Result
1	F + mAb + biotin + ABC-P + DAB	+
2	F + **B** + mAb + biotin + ABC-AP + DAB	+++
3	F + B + **Dp** + mAb + biotin + ABC-P + DAB	-
4	F + B + mAb + biotin + **Dp** + ABC-P + DAB	+++
5	F + mAb + biotin + ABC-AP + Vector red (no levamisole)	+
6	F + **B** + mAb + biotin + ABC-AP + Vector red (no levamisole)	+++
7	F + B + mAb + biotin + ABC-AP + Vector red (no levamisole)	+
8	F + B + mAb + biotin + ABC-AP + Vector red **(levamisole)**	+++
9	F + B + mAb + biotin + Dp + ABC-P + **DAB**	++++
10	F + B + mAb + biotin + Dp + ABC-P + **DAB-$NiCl_2$**	++
11	F + B + mAb + biotin + ABC-AP + **Vector red (levamisole)**	++
12	F + B + mAb + biotin + Dp + ABC-P + DAB **4°C**	+
13	F + B + mAb + biotin + Dp + ABC-P + DAB **22°C**	+++
14	F + B + mAb + biotin + Dp + ABC-P + DAB **37°C**	++

ABC-AP: avidin biotin alkaline phosphatase complex; ABC-P: avidin biotin peroxidase complex; B: block; DAB: diaminobenzidine 3,3' tetrahydrochloride; DAB-NiCl2: diaminobenzidine 3,3' tetrahydrochloride/ nickel chloride substrate; Dp: deperoxidation; F: fixation; mAb: primary monoclonal antibody

Table 2.6 Combinations of double staining used during preliminary studies in order to determine optimal staining procedure.

	Double staining comparisons made	Result
1	F + 1st mAb + biotin + ABC-P + DAB /wash/ 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+
2	F + **Bp** + 1st mAb + biotin + ABC-P + DAB /wash/ 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+++
3	F + Bp + 1st mAb + biotin + ABC-P + DAB /wash/ 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+++
4	F + Bp + 1st mAb + biotin + **Dp** + ABC-P + DAB /wash/ 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+
5	F + Bp + 1st mAb + biotin + ABC-P + DAB /wash/ acetic acid + 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+
6	F + Bp + 1st mAb + biotin + ABC-P + DAB **/KI/** + 2nd mAb + biotin + ABC-AP + Vector red (levamisole)	+++

ABC-AP: avidin biotin alkaline phosphatase complex; ABC-P: avidin biotin peroxidase complex; B: block; DAB: diaminobenzidine 3,3' tetrahydrochloride; Dp: deperoxidation; F: fixation; mAb: primary monoclonal antibody

(5) DAB (3, 3' Diaminobenzidine tetrahydrochloride) substrate gave the best staining with vector red and DAB-$NiCl_2$ giving slightly less clear staining (combinations 9-11);

(6) Finally the optimal temperature for ABC-P staining was 22°C (combination 12-14).

2.7.4.3 Double staining comparisons

The results from the double staining preliminary studies indicated (Table 2.6):

(1) Comparison 1 and 2 indicated that blocking before the first primary monoclonal antibody gave better staining than not blocking at all;

(2) Deperoxidation after the addition of biotin gave better staining (comparisons 3 and 4);

(3) Chaotropic agent (potassium iodide) used between the two staining methods gave better staining than just washing (comparisons 5 and 6);

(4) Non-specific staining and cross-reactivity between the two detection systems could not be eliminated.

2.7.4.4 Choice of staining method following preliminary studies

ABC-P method using DAB as the substrate gave the best staining results in comparison to ABC-P/DAB-$NiCl_2$, ABC-AP and double staining. Therefore the optimal staining method for these studies was ABC-peroxidase and if two antigens were of interest within the same section then serial

sections were used to determine if they were localised within the same area.

2.7.4.5 ABC-peroxidase (ABC-P) method

Tissue sections were stained using the ABC method of Hsu, Raine & Fanger, (1981) and is outlined in detail in Table 2.7. Briefly, sections were processed using seven major steps,

(a) Fixation - Frozen cryostat sections of the specimens were air dried and fixed in cold acetone;

(b) Blocking - Following fixation the sections were blocked with horse serum (section 2.4.4.4) to decrease non-specific background staining;

(c) Primary antibody - Unlabelled primary monoclonal antibodies were added at optimal dilutions and sections incubated at room temperature (Table 2.8);

(d) Biotinylated antibody - Biotinylated horse anti-mouse secondary antibody (section 2.4.4.5) was used to label the primary monoclonal antibodies;

(e) De-peroxidation - The sections were deperoxidised to remove endogenous peroxidase using methanolic hydrogen peroxide (section 2.4.4.6);

(f) ABC complex - Preformed avidin-biotinylated peroxidase complex (section 2.4.4.7) was then added;

(g) Reaction development - The reaction was developed using the substrate DAB (section 2.4.4.8) and staining enhanced by incubation of the sections in 0.5% $CuSO_4$ solution (section 2.4.4.15) before counterstaining with

Table 2.7 Immunostaining Procedure for Avidin-Biotin-complex - peroxidase (ABC-P) technique.

1*	Frozen cryostat sections air dried (60 mins)
2*	Immediately before staining, fix sections with cold acetone (BDH, UK) (10 mins)
3*	Transfer slides into PBS (3 changes) (10 mins)
4	Block sections with diluted normal horse serum (Vector Laboratories, UK) (20 mins)
5	Blot excess serum from sections
6	Incubate sections with primary monoclonal antibody (mouse anti-human) diluted in PBS for 60 mins
7	Wash slides in 3 changes of PBS (10 mins)
8	Incubate sections with biotinylated secondary antibody (horse anti-mouse IgG (H and L) (Vector Laboratories, UK) diluted in PBS for 30 mins
9	Wash slides in 3 changes of PBS (10 mins)
10	Incubate the sections in methanolic hydrogen peroxide (0.3%) for 30 mins
11	Wash slides in 3 changes of PBS (20 mins)
12*	Incubate sections with avidin-biotin-peroxidase complex[1] (ABC) (Vector Laboratories, UK) for 45 mins
13	Wash slides in 3 changes of PBS (10 mins)
14	Incubate sections in substrate[1] (0.01% H_2O_2 and 0.5% diaminobenzidine tetrahydrochloride) (DAB) in PBS pH 7.4 for 5-10 mins
15	Wash slides in PBS (5 mins) followed by distilled water (5 mins)
16*	Incubate sections in 0.5% copper sulphate solution for 5 mins
17	Wash slides in PBS (5 mins) followed by distilled water (5 mins)
18	Counterstain, dehydrate and mount

*Modifications to Hsu, Raine & Fanger, (1981[a]) method.
[1]make up 30 mins prior to use.
All incubations carried out at room temperature and in a moist chamber.

Table 2.8 Monoclonal antibodies optimal dilution used in the immunohistochemistry procedures. The source of the monoclonal antibody and the antigen it is directed against is indicated.

mAb	Antigen	Source	Optimal dilution	Reference
OKT6	CD1a	Ortho[1]	1/50	Harrist *et al.*, 1983 Fithian *et al.*, 1981
OKT3	CD3	A.T.C.C.	1/50	Hoffman *et al.*, 1980
UCHT-1	CD3	S.A.P.U.	1/4	
OKT3	CD3	Ortho	1/50	
OKT4	CD4	A.T.C.C.	1/50	Hoffman *et al.*, 1980
RFT4	CD4	S.A.P.U.	1/4	
OKT8	CD8	Ortho	1/50	Hoffman *et al.*, 1980
RFT8	CD8	A.T.C.C.	1/50	
OKT8	CD8	S.A.P.U.	1/4	
TS1/22	CD11a	Donation[2]	1/20	Sanchez-Madrid *et al.*,1982
2H4	CD45RA	Coulter	1/50	Morimoto *et al.*, 1985
UCHL-1	CD45RO	Donation[2]	1/25	Smith *et al.*, 1986
UCHL-1	CD45RO	Donation[3]	1/25	Norton *et al.*, 1986
6.5B5	CD54	Donation[2]	1/30	Wellicome *et al.*, 1990
1.2B6	ELAM-1	Donation[2]	1/20	Wellicome *et al.*, 1990
1.4C3	VCAM-1	Donation[2]	1/10	Wellicome *et al.*, 1990
EN4	EC	Seralab	1/100	Cui *et al.*, 1983
RFDR1	HLA-DR	S.A.P.U.	1/4	Lampson & Levy 1980
NP57	PMN elastase	Dako	1/300	Pulford *et al.*, 1988

[1] Orthodiagnostics
[2] Dr D.O. Haskard (London, UK)
[3] Prof. P.C. Wilkinson (Glasgow, UK)

Mayer's haematoxylin (section 2.4.4.16). One section from each biopsy was also stained with haematoxylin and eosin (section 2.7.4.8).

Monoclonal antibody specificities were confirmed using human tonsil and human skin as control tissue and appropriate negative control sections treated with nonspecific primary antibodies.

2.7.4.6 ABC-alkaline phosphatase (ABC-AP) method

The basic principals of this method are identical to the ABC-P method (section 2.7.4.5) except the substrate differs. The ABC-AP method is outlined in Table 2.9, the sections were fixed in cold acetone, blocked with horse serum, labelled with primary monoclonal antibody followed by biotinylated horse anti-mouse secondary antibody. ABC-AP complex was then added (section 2.4.4.12) and the reaction developed using the commercially available Vector Red(R) (section 2.4.4.13) containing 0.1mM levamisole (section 2.4.4.11) to remove endogenous alkaline phosphatase.

2.7.4.7 Double staining method

The double staining method was employed in an attempt to stain two antigens within the same tissue section i.e. ELAM-1 and PMN-elastase. The two detection systems utilised were ABC-P and ABC-AP. The ABC-P method was used initially to pick up the first primary monoclonal antibody

Table 2.9 Immunostaining Procedure for Avidin-Biotin-complex - alkaline phosphatase (ABC-AP) technique.

1	Frozen cryostat sections air dried (60 mins)
2	Immediately before staining, fix sections with cold acetone (BDH, UK) (10 mins)
3	Transfer slides into PBS (3 changes) (10 mins)
4	Wash slides in 3 changes of PBS (10 mins)
5	Block sections with diluted normal horse serum (Vector Laboratories, UK) (20 mins)
6	Blot excess serum from sections
7	Incubate sections with primary monoclonal antibody (mouse anti-human) diluted in PBS for 60 mins
8	Wash slides in 3 changes of PBS (10 mins)
9	Incubate sections with biotinylated secondary antibody (horse anti-mouse IgG (H and L) (Vector Laboratories, UK) diluted in PBS for 30 mins
10	Wash slides in 3 changes of PBS (10 mins)
11	Incubate sections with avidin-biotin-peroxidase - AP complex[1] (ABC-AP) (Vector Laboratories, UK) for 45 mins
12	Wash slides in 3 changes of PBS (10 mins)
13	Incubate sections in AP-substrate kit I vector red (made up in 100mM Tris-HCl, pH 8.2) for 15-20 mins in the dark.
14	Wash slides in distilled water (5 mins)
15	Counterstain, dehydrate and mount

[1]make up 30 mins prior to use.
All incubations carried out at room temperature unless otherwise stated and in a moist chamber.

(i.e. ELAM-1) and the ABC-AP kit used to label the second primary monoclonal antibody (i.e. PMN-elastase). Basically the ABC-P method as outlined in Table 2.7 was followed until step 15, then the ABC-AP method (Table 2.9) was followed beginning at step 3 in order to stain for the second antigen. Potassium iodide (0.06M, 30 mins, RT) (section 2.4.4.14) a chaotropic agent was used between the two methods in order to prevent cross-reaction between the two detection systems. Appropriate staining for ELAM-1 and PMN-elastase would stain ELAM-1^{+} blood vessels brown and PMN-elastase positive cells red.

2.7.4.8 Haematoxylin and eosin sections

Tissue sections were thoroughly air-dried and then placed in Mayer's haematoxylin (section 2.4.4.16) for 10 minutes, rinsed under running water, then placed in 0.1% eosin (section 2.4.4.17) for 10 seconds. Sections were finally dehydrated in a range of alcohols and mounted under xylene.

2.7.5 Primary monoclonal antibodies

A range of primary mAbs were used to stain the gingival tissue (Table 2.8). All mAbs were mouse anti-human and of various isotypes. Optimal dilutions for the mAbs were pre-determined by titration before use in the ABC technique (Table 2.8).

2.7.6 Enumeration

2.7.6.1 Areas

The experimental gingival biopsies were divided into four areas for enumeration as shown in Figure 2.7. An imaginary line (AB) was dropped from the crest of the gingiva to divide the tissue into two halves - the junctional epithelium/sulcular epithelium (JE/SE) and the oral epithelium (OE). A second imaginary line was drawn horizontally (CD) just below the termination of the JE perpendicular to the first line AB (interception point - E) (Fig. 2.7). The four areas were labelled as follows,

(i) JE/SE - junctional epithelium/sulcular epithelium,
(ii) OE - oral epithelium,
(iii) JE/CT - connective tissue subjacent to the JE,
(iv) OE/CT - connective tissue subjacent to the OE.

For enumeration the methods employed were cell counting, grading of the leucocyte infiltrate size, grading of blood vessel staining intensity and also calculation of the percentage of adhesion molecule positive vessels (percentage positivity) compared to the total number of vessels.

2.7.6.2 Grading of overall cellular infiltrate

Haematoxylin and eosin sections were viewed under low magnification (x100) and cellular infiltrate scored as +: very little infiltration; ++: small infiltrate; +++ moderate infiltration and ++++: large infiltrate (Fig. 2.7a)

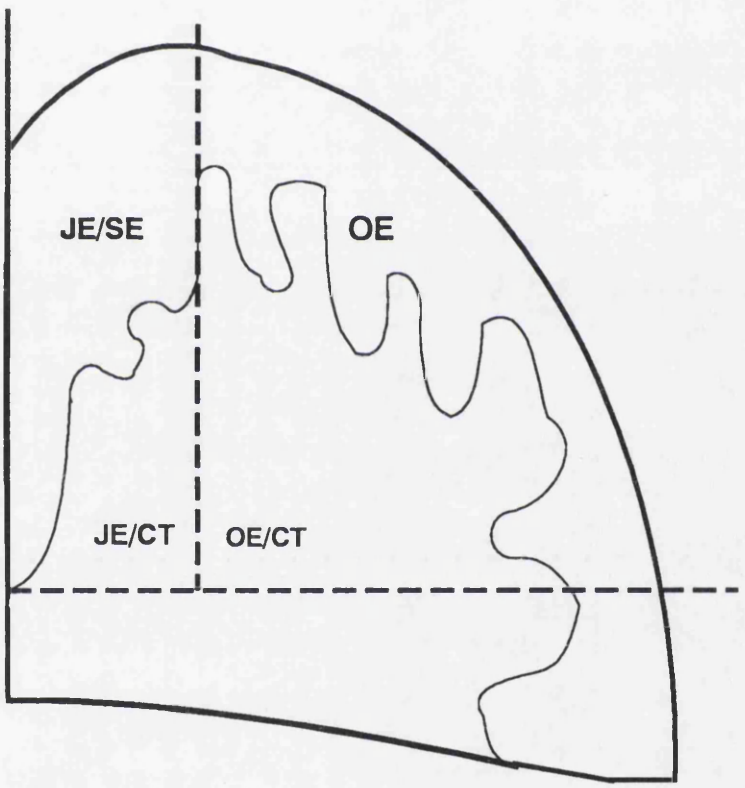

Figure 2.7 Areas used for enumeration, junctional/sulcular epithelium (JE/SE); oral epithelium (OE); connective tissue subjacent to junctional/sulcular epithelium (JE/CT) and connective tissue subjacent to oral epithelium (OE/CT).

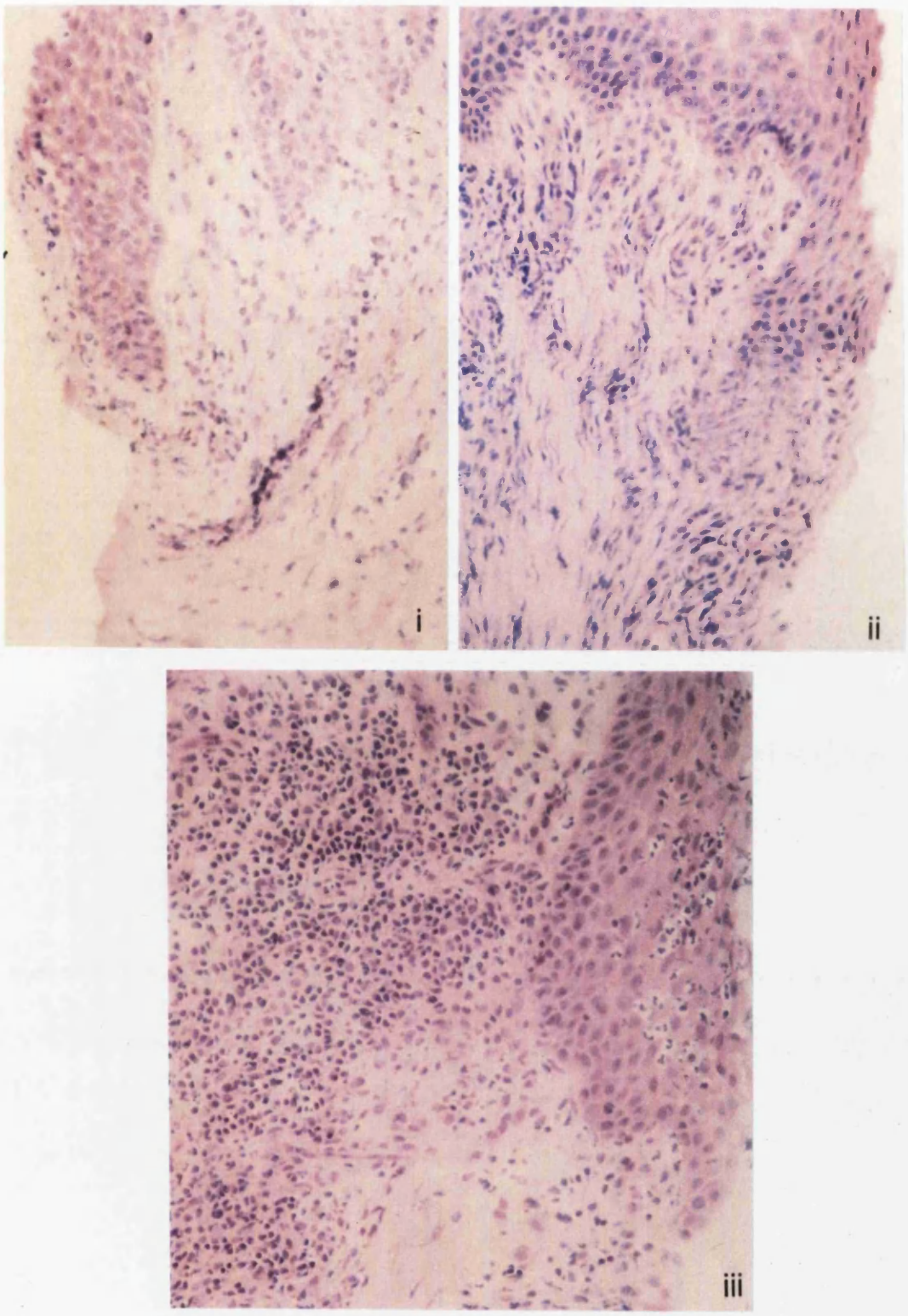

Figure 2.7a Shows a pictorial representation of the grading used to enumerate the cellular infiltration; **(i)** graded + very little infiltration, **(ii)** graded ++ small infiltrate, and **(iii)** +++ moderate infiltrate (H&E, mag. x200).

2.7.6.3 Counting method

Positive cells within the four areas (section 2.7.6.1) were counted using an ocular grid (5 x 5). Using computer assisted image analysis (Video Vector Dynamics, Glasgow UK) the 5x5 ocular grid was superimposed onto an S8-stage micrometer (1mm/0.01 divisions) (Graticules Ltd, Kent, UK) to measure the length of 5 squares and depending on the magnification used the scale was then entered into the computer (Table 2.10). Using high power magnification (x400) and a light cursor the area covered by the inner nine squares of the ocular grid was measured and a fixed counting area established (area = field = 0.018mm^2). The ocular grid was superimposed onto the section to be counted and positive cells within the area covered by the inner nine squares (=field) were counted.

For Langerhans cell enumeration (CD1a$^+$ or HLA-DR$^+$) 10 high power fields (x400 magnification, area=0.018mm^2) within the OE were counted for each biopsy minimum of 3 sections per biopsy taken during the experimental gingivitis studies and results expressed as the mean number of positive cells per high power field (n=10 fields). For the remaining leucocytes 3 high power fields were counted within the OE and JE/SE and results expressed as number of positive cells per mm^2.

For the two connective tissue areas (JE/CT and OE/CT) positive cells were counted in the most densely infiltrated

Table 2.10 Scaling used during computerised image analysis enumeration of positive cells. Lengths of 5 squares on ocular grid depending on the magnification used.

Magnification	Scale
x10	905μm
x16	570μm
x25	368μm
x40	226μm

field within the area, with only one field counted in any one section (maximum n=3 sections). For each biopsy, results were expressed as the mean number of positive cells per mm^2 (n=3 fields).

All fields were chosen randomly and counting was performed 'blind' i.e. counted without prior knowledge of experimental treatment (days of plaque accumulation).

2.7.6.4 Grading of leucocyte infiltrate

To grade leucocyte infiltration of the tissue semi-quantitative ordinal scales were used. For PMN infiltration the whole area was taken as the unit of area and PMN infiltrate was graded as 1 if 1-10 cells were present in the unit area, 2; 11-30 cells, 3; 31-100 cells and 4;)100 cells in the area unit. For remaining leucocytic infiltration a slightly different ordinal scale was used (1; 1-10 cells, 2; 11-20 cells, 3; 21-75 cells and 4; >75 cells), with cells being graded within high power (x400 magnification) connective tissue fields in the most densely infiltrated area.

2.7.6.5 Grading of blood vessel staining intensity

Counting positive cells is relatively straight forward, if a cell is positive it is counted, if it is negative then it is ignored. Staining of blood vessels on the other hand can cause problems with enumeration as positive vessels can vary in length and therefore can give false results.

Grading staining intensity and calculating the percentage positive vessels can overcome and reduce the possibility of false results.

Blood vessel staining intensity was graded semi-quantitatively on a 4-point scale using the method of Messadi *et al.* (1987) (Table 2.11) (Fig. 2.7b). Keratinocyte staining was graded in a similar fashion: - no staining; -/+ very weak staining; + weak staining; ++ moderate staining; +++ strong staining and ++++ very strong staining. Vessels were graded within JE/CT and OE/CT areas (ELAM-1, ICAM-1 and VCAM-1) and ICAM-1 staining of the keratinocytes within OE, SE and JE when present. Each section was assessed twice in random order and 'blind'. This method of quantification was reproducible as shown by the following Spearman's rank correlation coefficients between the two readings of the 20 biopsies (21-day study); ELAM-1 CT r=0.800: ICAM-1 CT r=0.915: ICAM-1 SE keratinocyte r=0.864 and ICAM-1 JE keratinocyte r=0.829.

2.7.6.6 Calculation of the percentage of adhesion molecule positive vessels

Percentage positive vessels were calculated using EN4 monoclonal antibody (Seralab, UK) as a positive control. Serial sections were stained with EN4 mAb and adhesion mAbs (ELAM-1, ICAM-1 and VCAM-1). High power fields were chosen in adjacent serial sections and the number of positive vessels counted. The percentage of adhesion molecule

Table 2.11 Messadi *et al.* (1987) grading scale.

-	no staining
+	weak focal granular staining
++	moderate staining of vessels
+++	strong staining of vessels
++++	very strong staining of vessels

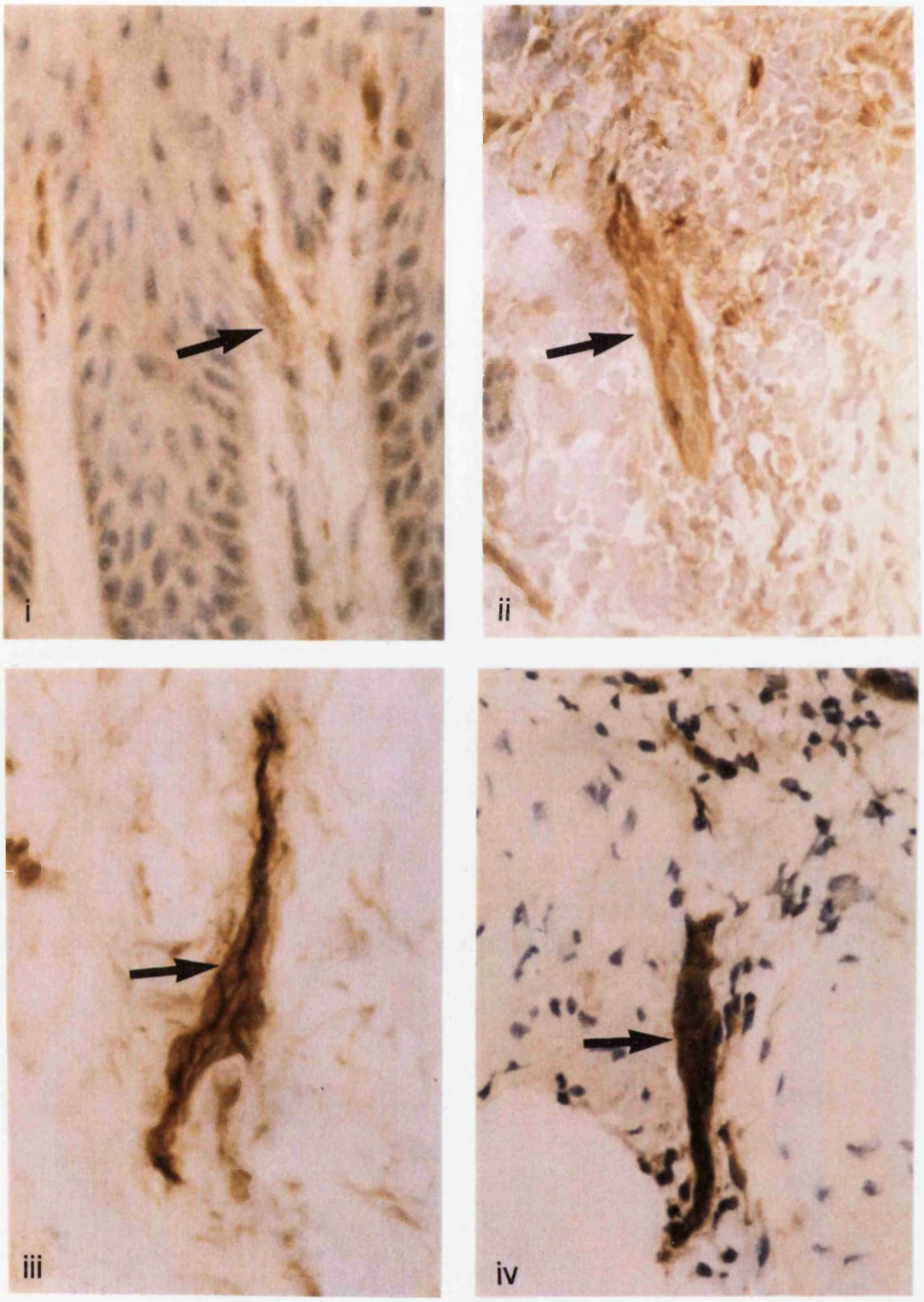

Figure 2.7b Shows a pictorial representation of Messadi *et al.*'s (1987) grading scale, **(i)** graded + weak staining, **(ii)** graded ++ moderate staining, **(iii)** graded +++ strong staining, and **(iv)** graded ++++ very strong staining (solid arrows) (Immunoperoxidase, mag. x500).

positive vessels were then calculated as,

$$\% \text{ positive vessels} = \frac{\text{number of adhesion mAb positive vessels}}{\text{number of EN4 positive vessels}} \times 100$$

Three high power fields were counted per biopsy and results expressed as the mean percentage positive vessels.

2.7.6.7 Presentation of organ culture results

The percentage of adhesion molecule positive vessels was calculated as discussed previously (section 2.7.6.6) and grading of the intensity of staining within the connective tissue subjacent to the epithelium was performed as outlined in section 2.7.6.5. The results were expressed in two formats;

(i) The results for the blocks cultured in control media were expressed as the percentage of positive vessels per block or as overall staining intensity.

(ii) For blocks cultured in cytokine-enriched media (section 2.8.2) the results were expressed as the net change in the percentage of positive staining vessels, corrected for background changes and the net changes in staining intensity. Thus the overall change in the percentage positivity was calculated as,

$$\% \text{ net change} = \text{cytokine } \% \text{ positive} - \text{control } \% \text{ positive}$$

and the overall change in vessel staining intensity calculated as,

net change staining intensity = cytokine intensity - control intensity.

2.7.7 Statistical analysis

A variety of statistical procedures were used depending on the type of sample collected (repeated measures, paired t-tests, two sample, pooled t-test) and the type of analysis required.

2.7.7.1 Repeated measures analysis of variance (MANOVA)

For the experimental gingivitis study the leucocyte infiltrate were obtained from the same individual at regular time points and this thus constitutes a repeated measures design. The data followed a normal distribution and were analysed using the repeated measures analysis of variance (MANOVA) using the statistical package SPSS on an IBM personal computer.

If the MANOVA test was statistically significant ie if 'day' had a statistically significant effect on changes in leucocyte number then paired t-tests were used to further analyse where these changes were occurring. Since multiple comparisons were used, a Bonferroni correction was needed. The Bonferroni correction employed was calculated as, probability value (0.05) divided by the number of possible comparisons (n=6). The acceptable level therefore is $p<0.01$.

2.7.7.2 Friedman's test

Repeated measures data on grading of adhesion molecule intensity obtained from the experimental gingivitis study, collected on an ordinal scale and hence usually gave a non-normal distribution were analysed using the Friedman's test (Minitab PC), which is the non-parametric equivalent of the MANOVA. If the Friedman's test is statistically significant, then further analysis is used to decide which population is different. The test used was determined by the distribution of the differences between the populations, if the difference is normally distributed then the Wilcoxon signed rank test is used (Minitab PC), if the distribution of the difference is non-normally distributed then the sign test is used (Minitab PC).

2.7.7.3 Correlation

To demonstrate association between grading of staining intensity between areas, two different correlations were used. Pearson's correlation coefficient was used if the samples demonstrated a normal or Gaussian distribution and Spearman's rank correlation if the distribution was skewed. Both analyses were carried out on using the 'Minitab' statistical package on an IBM PC.

2.8 Organ culture study

2.8.1 Tissue source

Tissue for the organ culture studies was obtained as mentioned previously (section 2.2.2). After collection the

tissue was immediately placed on gauze and transported to the laboratory where it was set up in organ culture within 30 mins of its removal from the mouth.

2.8.2 Organ culture method

Tissue samples were washed in several changes of HBSS (section 2.4.5.1) in order to remove adherent blood clots before being sectioned to 1mm cubes. The explants were then transported with the connective tissue side down onto lens paper and placed on stainless steel grids within the organ tissue culture (OTC) dishes (Falcon Plastics/Becton Dickinson, UK Ltd) (Fig. 2.8) (Bernstein, Preisig & Schroeder, 1988) with 0.75 ml of medium 199 (section 2.4.5.2) containing 0.1% BSA (Sigma, UK) (control) or cytokines IL-1β, TNFα or LPS or rhIFγ (sections 2.2.5.3 - 2.2.5.7 respectively) was added to each OTC dish to the level of the steel grid. Cultures were maintained at 37°C in a humidified incubator in 5% CO_2 in air for varying times ranging between 6-72 hours. The culture medium was not replaced during the culture period. Pre-incubation controls were prepared in the manner described above, but were immediately removed from the culture dish before the addition of the culture medium. After incubation the explants were removed from the OCT dishes and blotted on filter paper to remove excess fluid and then embedded in tissue tek OCT and immediately frozen. Eight micrometer sections were collected on vectabond treated slides, allowed to air dry and then stored at -70°C. The sections

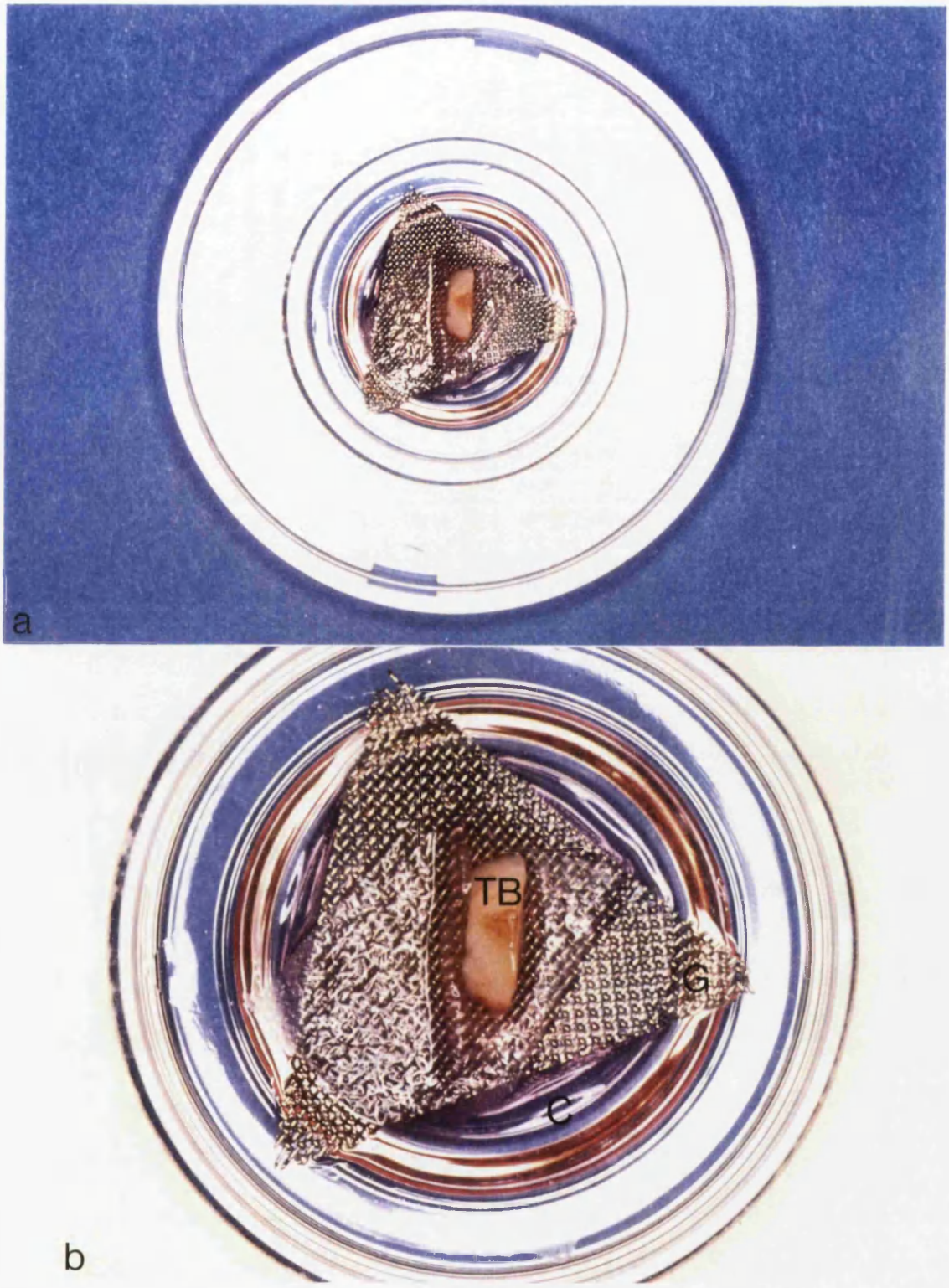

Figure 2.8 (a) Organ culture dish setup. **(b)** Closeup of well showing tissue block (TB) separated from the cytokine source (C) by a metal grid (G).

were then processed using the ABC method previously described (section 2.7.4.5).

2.8.3 Vessel enumeration

The percentage of adhesion molecule positive vessels were calculated as discussed previously (section 2.7.6.6) and intensity of blood vessel staining was determined within the subjacent connective tissue (section 2.7.6.5).

Chapter 3

Results

3.1 Experimental gingivitis studies

The experimental gingivitis model (Löe *et al.*, 1965) was used to study the development of gingival inflammation. Two studies were performed. In the first study inflammation was induced experimentally by abolishing oral hygiene procedures for 21 days and in the second study this period was reduced to 10 days.

3.1.1 Twenty-one day experimental gingivitis study

Six individuals (A-F) commenced this study and five fully completed the study (A-E) (section 2.2.1). Clinical indices, GCF samples and gingival biopsies were collected and processed as previously described.

3.1.1.1 Clinical indices

At baseline i.e. in clinically 'healthy' gingiva, all subjects had minimal plaque (PI=0) except subject B who had PI=1 at one site and no clinically visible gingival inflammation (MGI=0) (Fig. 3.1). Over the 21 day experimental period the PI of the biopsy sites rose and the rise in the MGI lagged behind by a few days. Both indices increased significantly with time (PI, $p=0.002$ and MGI, $p=0.001$) (Friedman's test, Minitab) and further analysis demonstrated that indices were significantly higher at day 14 and day 21 when compared with day 0 (Wilcoxon Sign Rank Test, Minitab).

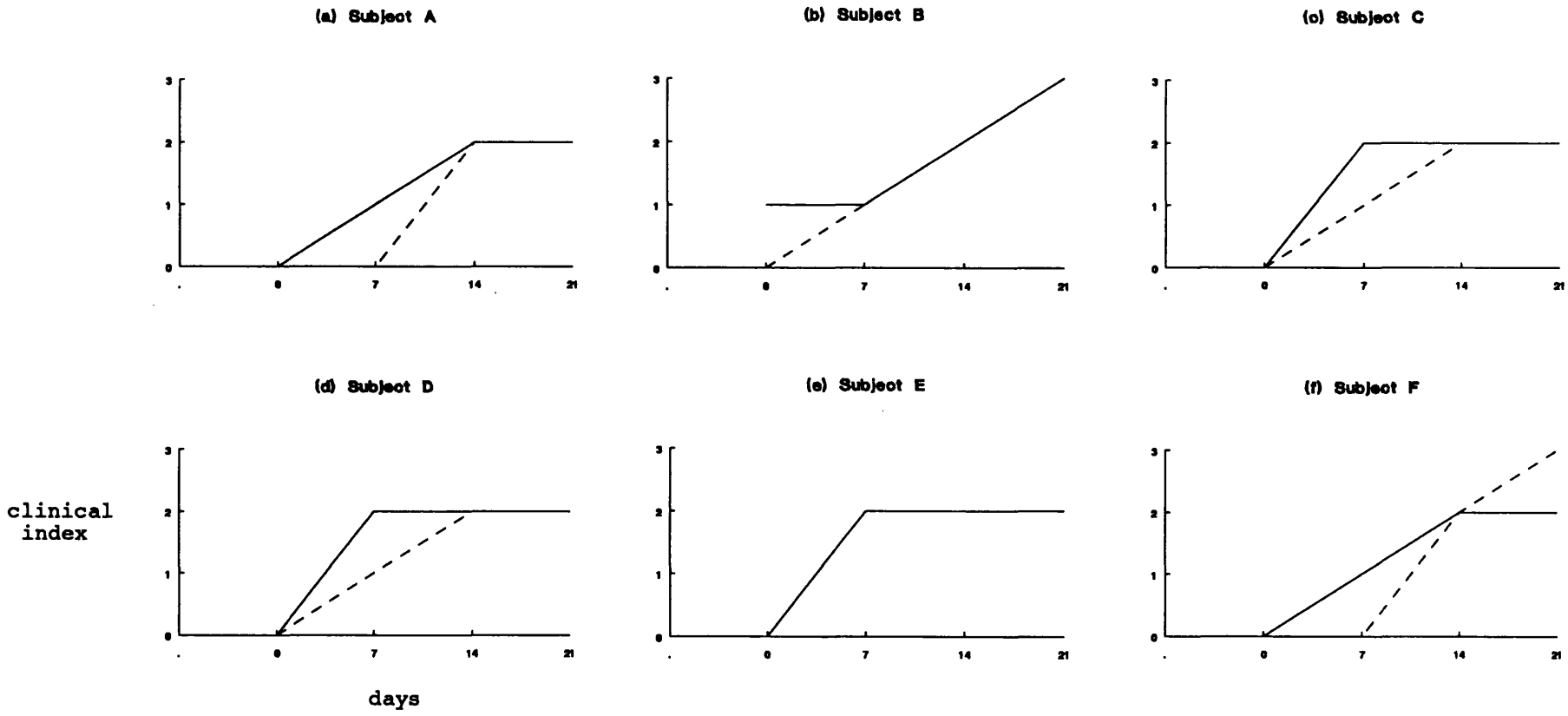

Figure 3.1 Clinical index scores for the mid-buccal area (i.e. the biopsy site) obtained during the 21-day experimental gingivitis study. Graphs (a)-(f) show individual site scores for the plaque index (PI) (——) and the modified gingival index (MGI) (---) for subjects A-F respectively. The x-axis and y-axis legends for all the graphs are identical.

Spearman's rank correlation showed that over the 21-day period the site PI and MGI demonstrated a strong relationship with each other and a slightly weaker correlation with the day of oral hygiene abstention (Table 3.1).

3.1.1.2 Haematoxylin and eosin sections

Clinically 'healthy' gingival tissue exhibited a wide range in the extent of cellular infiltrate (Table 3.2). Some subjects exhibited more cellular infiltrate than others (subject B compared with subject D), with no relationship between the size of cellular infiltration and days of plaque accumulation (Spearman's rank correlation coefficient, Minitab).

Histologically the clinically 'healthy' gingival tissue (day 0) was well structured, but as inflammation developed (day 7 to day 21) their was a marked change with rete peg proliferation, increased vascularisation as noted by the appearance of capillaries within the JE/SE and JE areas and also migration of cells into the JE/SE epithelium area. Overall with the development of inflammation their were marked histological changes.

3.1.1.3 ELAM-1 and ICAM-1 expression

Within clinically 'healthy' gingiva (day 0) (Subjects A-E) both ELAM-1 and ICAM-1 staining were seen (Tables 3.3 and 3.4 respectively). ELAM-1^{+} blood vessels were present in

Table 3.1 Regression analysis of clinical indices during the 21-day experimental gingivitis study. Spearman rank correlation coefficient (r) and probability values (p) are shown. Sites examined were the mid-buccal aspects of first molars i.e. the biopsy sites.

Parameters	r	p
PI vs Day	0.833	<0.001
MGI vs Day	0.893	<0.001
PI vs MGI	0.916	<0.001

MGI: Modified Gingival Index; PI: Plaque Index

Table 3.2 Size of cellular infiltration during the 21-day experimental gingivitis study.

Subject	Size of cellular infiltrate			
	Day 0	Day 7	Day 14	Day 21
A	+	++	+++	+
B	+++	+++	++	+++
C	+++	+++	+++	++
D	++	++	+	+
E	+	++	++	+++

Table 3.3 ELAM-1 staining intensity within JE/CT and OE/CT areas, for each subject at each sampling time during the 21-day experimental gingivitis study.

Day	Subject	Intensity of ELAM-1 staining	
		JE/CT	OE/CT
0	A	+++	++
	B	+++	++++
	C	++	+
	D	+++	+++
	E	+++	++
	median	+++	++
7	A	+	+
	B	+++	++
	C	+++	++
	D	+++	++
	E	++	+
	median	+++	++
14	A	*	*
	B	++	+++
	C	*	*
	D	+++	+
	E	++	+
	median	++	+
21	A	++++	+++
	B	+++	++
	C	*	*
	D	++	++
	E	++	+
	median	++/+++	++

* missing value

Table 3.4 ICAM-1 staining intensity for each subject at each time point during the 21-day experimental gingivitis study.

Day	Subject	Intensity of ICAM-1 staining				
		JE/CT	OE/CT	JE	OSE	OE
0	A	*	*	*	*	*
	B	+++	++	+++	++	-
	C	++	+	+++	++	-
	D	+++	++	+++	++	-
	E	+++	+++	+++	++	-
	median	+++	++	+++	++	-
7	A	++++	++++	++++	+++	-
	B	++	+	++	++	-
	C	+++	+++	+++	++	-
	D	*	*	+++	++	-
	E	+++	+++	+++	++	-
	median	+++	+++	+++	++	-
14	A	+++	++	+++	++	-
	B	*	*	+++	++	-
	C	++	+++	+++	+	-
	D	++	+++	++	++	-
	E	++++	++++	++	++	-
	median	++/+++	+++	++	++	-
21	A	+++	+++	++	++	-
	B	++++	+++	+++	++	-
	C	+++	++	++	+	-
	D	+++	+++	++	+	-
	E	+++	+++	++/+++	++	-
	median	+++	+++	++	++	-

* missing value

both the JE/CT and OE/CT of healthy gingiva (day 0), although expression was mainly concentrated in the JE/CT area (Fig. 3.2). As inflammation developed the concentration and intensity of staining in the JE/CT area became more marked as demonstrated by a 15% increase in the numbers of biopsies with stronger staining vessels at the JE/CT (day 21 compared with day 0) (Table 3.5).

ICAM-1 staining of blood vessels in the connective tissue also showed a similar distribution and pattern of staining, with a 20% decrease in the numbers of biopsies with stronger staining vessels at the JE/CT area (day 0 compared with day 21) (Table 3.5). Interestingly, clinically 'healthy' tissue also demonstrated intense ICAM-1 staining of the keratinocytes at the JE, with moderate staining of SE keratinocytes and no staining of OE keratinocytes (Fig. 3.3). With the subsequent development of inflammation there was little change in the intensity or distribution of ICAM-1 staining and no spread of ICAM-1 positivity to the OE keratinocytes (Table 3.4), although small parts of the keratinocytes within the stratum spinosum demonstrated very weak staining and on further analysis these exhibited the dendritic appearance of LCs (Fig. 3.4).

Throughout the 21 days the ICAM-1 staining of the JE keratinocytes appeared to demonstrate a gradient effect, with the most intense ICAM-1 staining towards the crevicular/tooth aspect and decreasing staining intensity

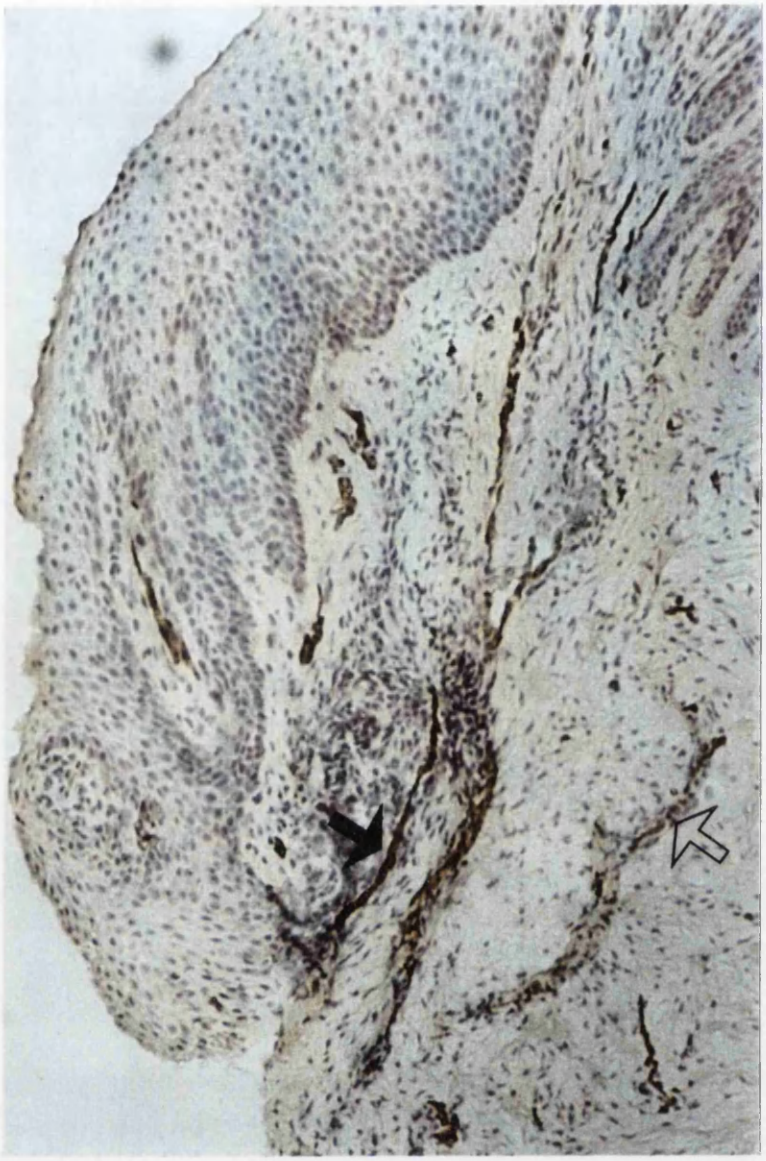

Figure 3.2 ELAM-1 positive vessels in the connective tissue area of a day 0 biopsy, the most intense staining ELAM-1^+ vessels located at the junctional epithelium/connective tissue area (solid arrow), with weaker staining ELAM-1^+ vessels located towards the oral epithelium/connective tissue area (open arrow) (Immunoperoxidase, mag. x125).

Table 3.5 Percentage of biopsies with stronger staining vessels at JE/CT compared with OE/CT within the same biopsies taken during the 21-day experimental gingivitis study.

	JE/CT > OE/CT			
	Day 0	**Day 7**	**Day 14**	**Day 21**
ELAM-1	60%	100%	66%	75%
ICAM-1	60%	20%	25%	40%

Figure 3.3 Intense ICAM-1 staining of junctional epithelium keratinocytes (solid arrow) with moderate staining of SE keratinocytes (open arrow) and relative absence of ICAM-1 staining of oral epithelium keratinocytes in a Day 7 biopsy (Immunoperoxidase, mag. x78.75).

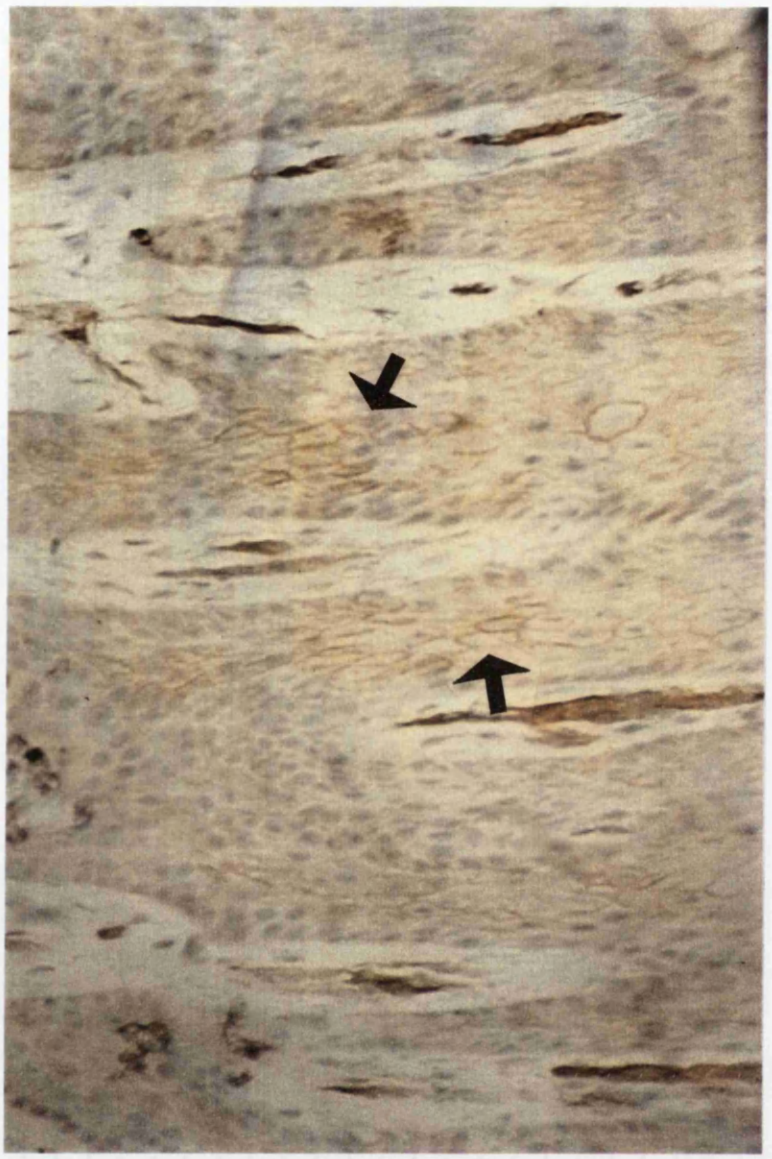

Figure 3.4 Day 0 biopsy demonstrating ICAM-1^{+} Langerhans cells within the oral epithelium (solid arrows) (Immunoperoxidase, mag. x200).

towards the basal layers (Fig. 3.5). Apical JE, when present, also expressed ICAM-1.

Since intensity was graded on an ordinal scale data analysis was by the repeated measures Friedman's Test (section 2.7.7.2). Due to technical difficulties full data for all four sampling times were available for only 3 of the 5 subjects for ELAM-1 intensity (subjects B, D, and E) and only 2 of the 5 subjects for ICAM-1 (subjects C and E). The Friedman's test demonstrated no significant day effect on ELAM-1 staining intensity within the two connective tissue areas studied (JE/CT and OE/CT), however there was a trend, which can be seen in Table 3.3, for ELAM-1 intensity to decrease with time (day 0 compared with day 21). Only two subjects possessed full data set for ICAM-1 therefore Friedman's analysis was not carried out. The lack of significant day effect may reflect the low numbers of subjects with complete data sets (n=3) in the analysis. Subject did have a significant affect on OE/CT ELAM-1 vessel staining ($p=0.037$) in that subject B tended to have higher staining intensity over the sampling period (Table 3.3).

Since JE/CT and OE/CT blood vessel staining differed within the same biopsies, the Spearman's rank correlation coefficient was used to measure the relationship between intensity of blood vessel staining in the two areas. ICAM-1 JE/CT and OE/CT staining (Spearman's rank correlation

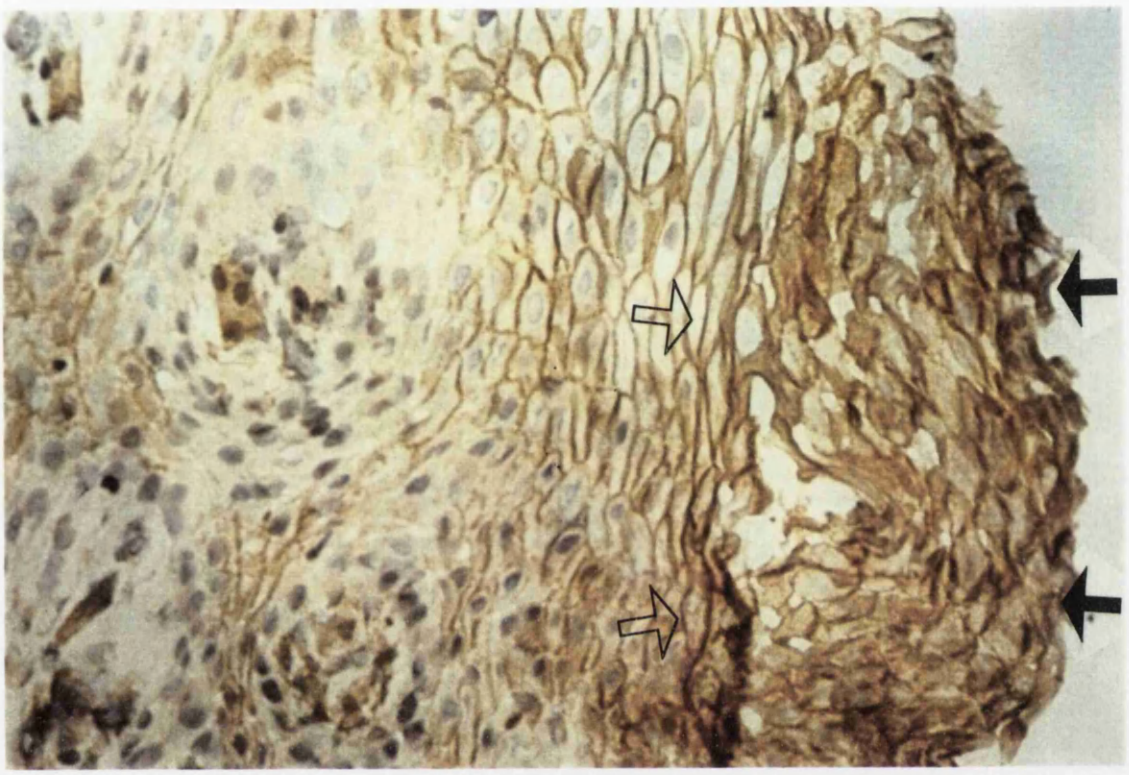

Figure 3.5 Day 7 biopsy showing an ICAM-1 gradient, with maximal staining at the junctional epithelium/tooth interface (solid arrows), with weaker staining in the basal layers of the junctional epithelium (open arrows) (Immunoperoxidase, mag. x312.5).

coefficient and Regression analysis; r=0.568, p=0.013) correlated just as well as ELAM-1 JE/CT and OE/CT (r=0.588, p=0.017) i.e. the biopsies which had high JE/CT staining tended to have high OE/CT staining (but lower than JE/CT) and vice-versa.

3.1.1.4 Neutrophils

PMN numbers over the 21 day period did not change significantly in the four areas of the gingival biopsy examined (JE/SE, OE, JE/CT and OE/CT) (MANOVA, SPSS) (Figs. 3.6 & 3.7). However as inflammation developed, there were significantly more PMNs present in the JE/CT than the OE/CT ($p<0.05$ in each case) (Mann-Whitney, Minitab). PMNs were not observed in the OE but were present at all time points in the JE. Furthermore, within the JE/SE PMNs were found to be more concentrated towards the gingival crevice (Fig. 3.8) except at day 7 when no PMNs were detected within the JE/SE.

PMNs when graded within specified gingival areas (section 2.7.6.4), and when compared with the intensity of ELAM-1 staining demonstrated weak associations between PMN grading and the grading of ELAM-1 staining within JE/CT and OE/CT (r=0.034 and r=0.280 respectively, Spearman's rank correlation coefficient).

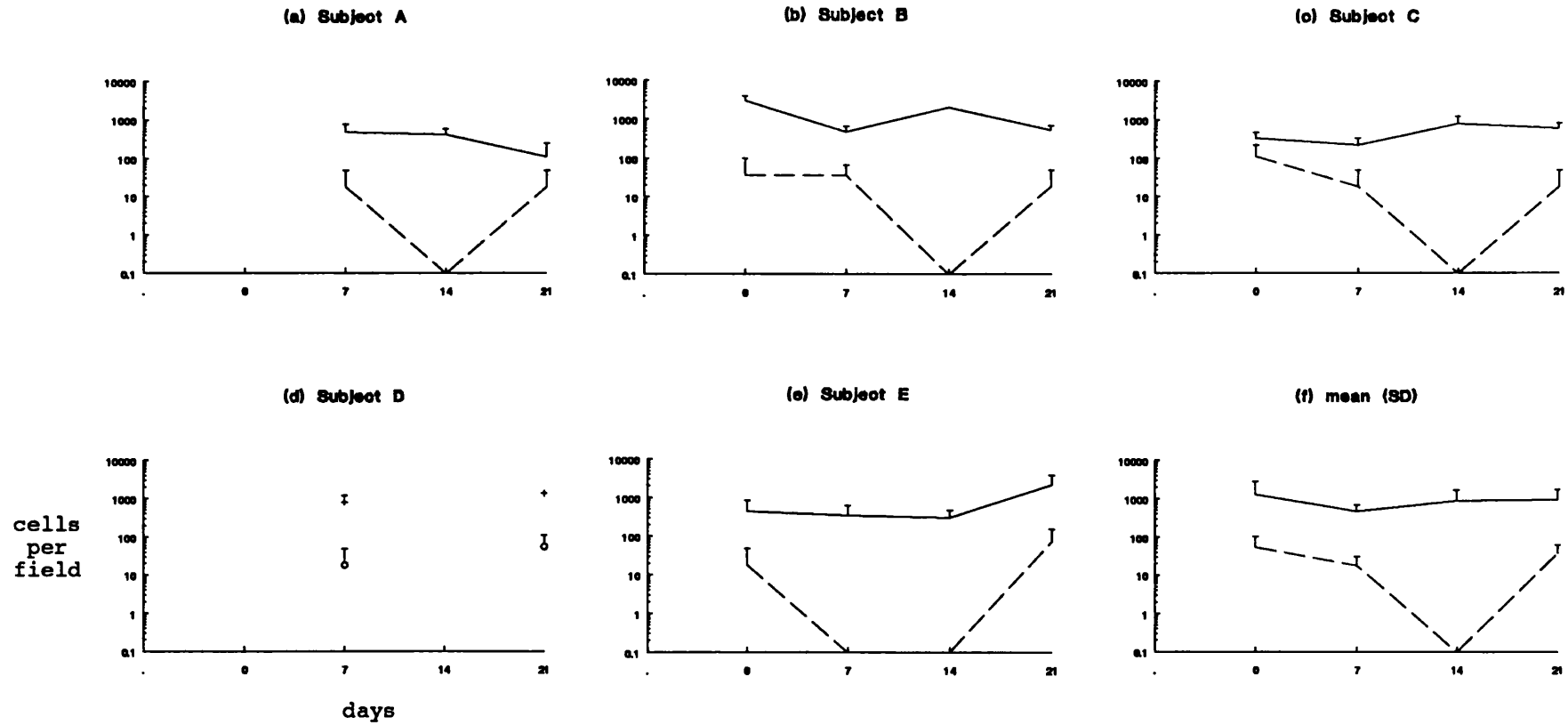

Figure 3.6 Graphs (a)-(e) show changes within neutrophil (PMN-elastase$^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (—— and +) and subjacent to the oral epithelium (OE/CT) (--- and ○) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

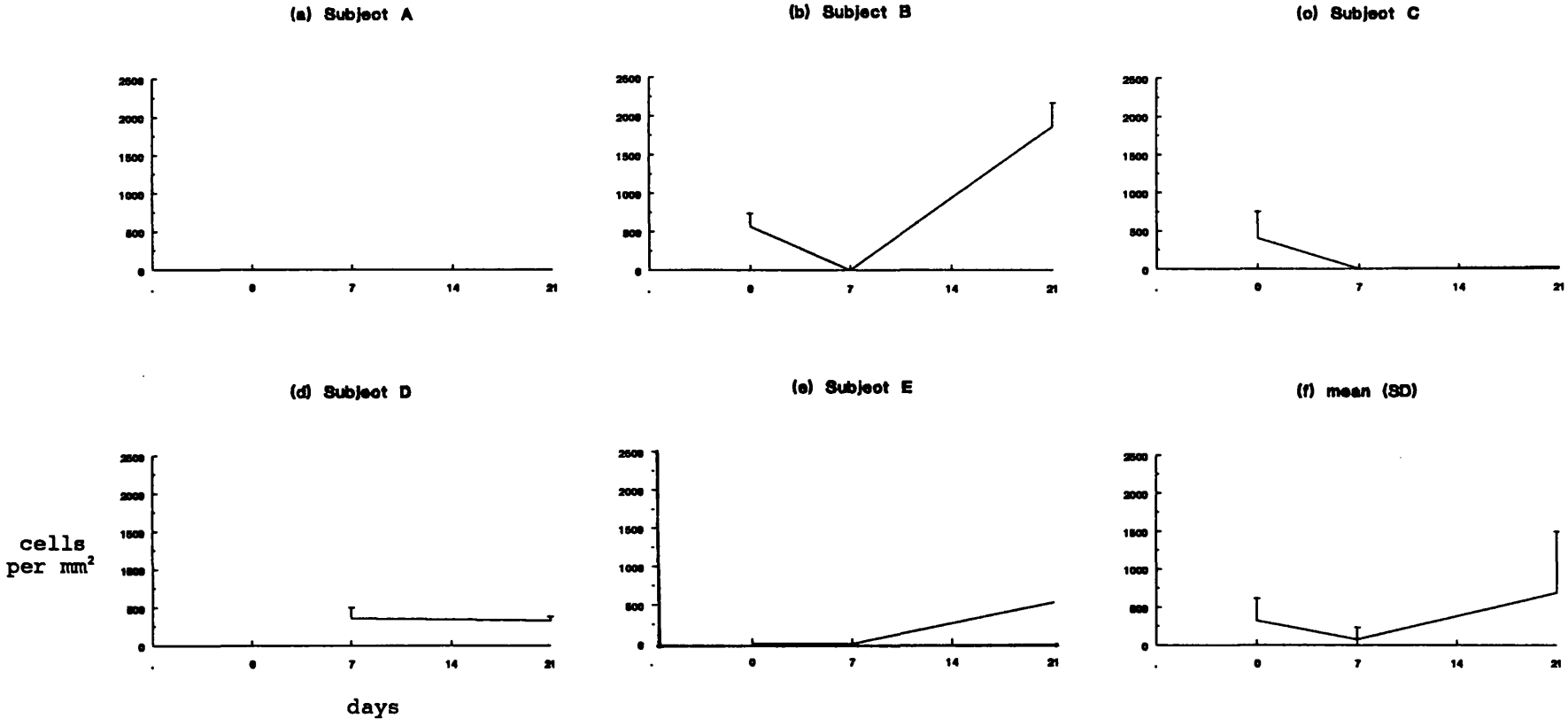

Figure 3.7 Graphs (a)-(e) show changes within neutrophil (PMN-elastase$^+$) numbers within the junctional epithelium/sulcular epithelium (JE/SE) (——) and oral epithelium (OE) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

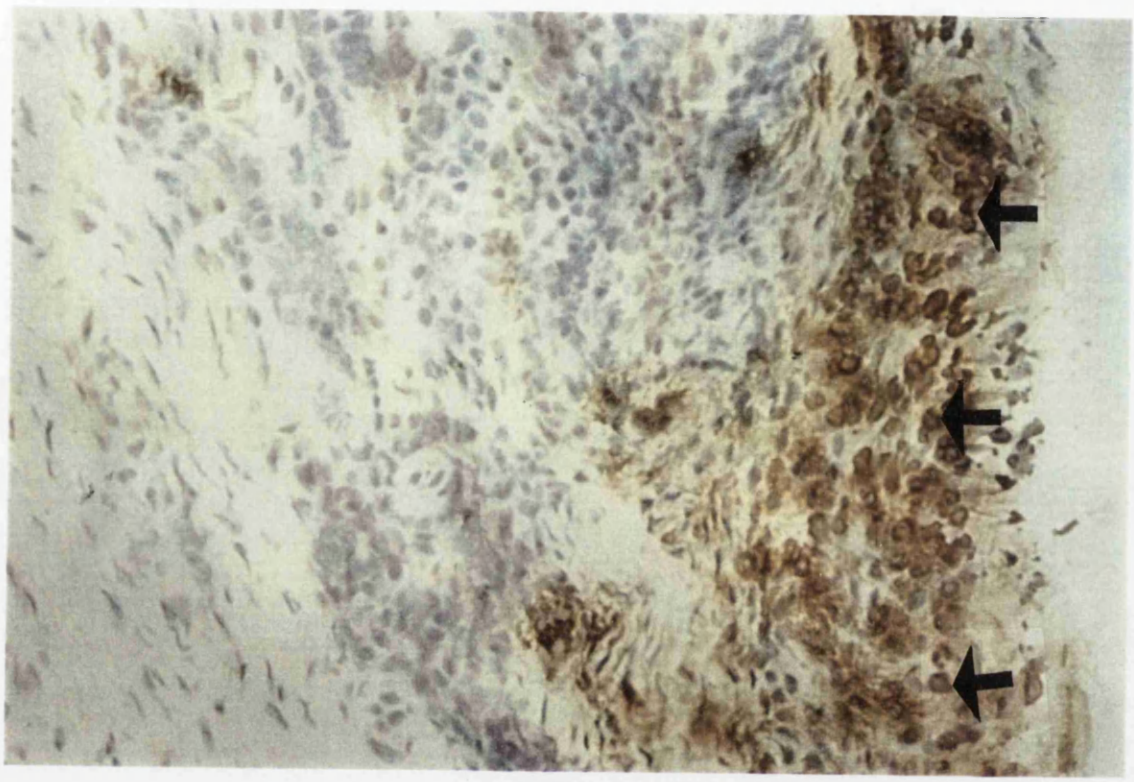

Figure 3.8 PMNs located at the junctional epithelium/tooth interface of a Day 0 biopsy (solid arrows) (Immunoperoxidase, mag. x312.5).

3.1.1.5 T cells

Within clinically 'healthy' tissue, cells which stained positive for the pan T cell marker CD3 were noted in abundance and with developing inflammation cells were noted in all areas (JE/SE, OE, JE/CT and OE/CT) and at all time points. $CD3^+$ cells appeared to be preferentially located within JE/CT when compared with OE/CT (Fig. 3.9) and within JE/SE when compared with OE (Fig. 3.10), with $CD3^+$ cells only significantly elevated after 14 days of plaque accumulation ($p=0.0122$) (Mann-Whitney Test, Minitab). Interestingly, when cells were noted within the OE/CT they appeared to cluster around the OE rete pegs (Fig. 3.11) and this feature was seen in all biopsies at all time points. Positive cells were also located within the epithelium. These intraepithelial lymphocytes (IEL) were basally located in clinically 'healthy' tissue (within the first cell layer just above the basement membrane) and with the development of inflammation the IELs shifted and were now seen within the basal, stratum spinosum and stratum granulosum layers of the OE (Fig. 3.12a). JE/SE contained numerous $CD3^+$ IELs (Fig. 3.12b).

T cell infiltrate was expressed as positive cells per mm^2. $CD3^+$ cells were present in all tissue compartments at all time points, with $CD3^+$ cells only being elevated significantly within OE over time ($p=0.024$, $n=4$ subjects with full data sets B, C, D and E, MANOVA, SPSS) and on further analysis (paired t-test, SPSS), $CD3^+$ cells were

	N	MEDIAN	Q1	Q3	95% CONFIDENCE INTERVAL
JECT D0	4	433	298	1511	(-441.7,1942.7)
JECT D7	5	3508	727	5672	(173.2,6349.2)
JECT D14	5	1610	813	5067	(-284.6,5632.6)
JECT D21	4	1873	468	3312	(-544.8,4313.3)

	N	MEDIAN	Q1	Q3	95% CONFIDENCE INTERVAL
OECT D0	4	102	36	411	(-170.8, 537.0)
OECT D7	5	477	233	1449	(-126.1,1662.1)
OECT D14	5	316	169	400	(142.9, 438.7)
OECT D21	4	413	335	949	(-27.8,1159.8)

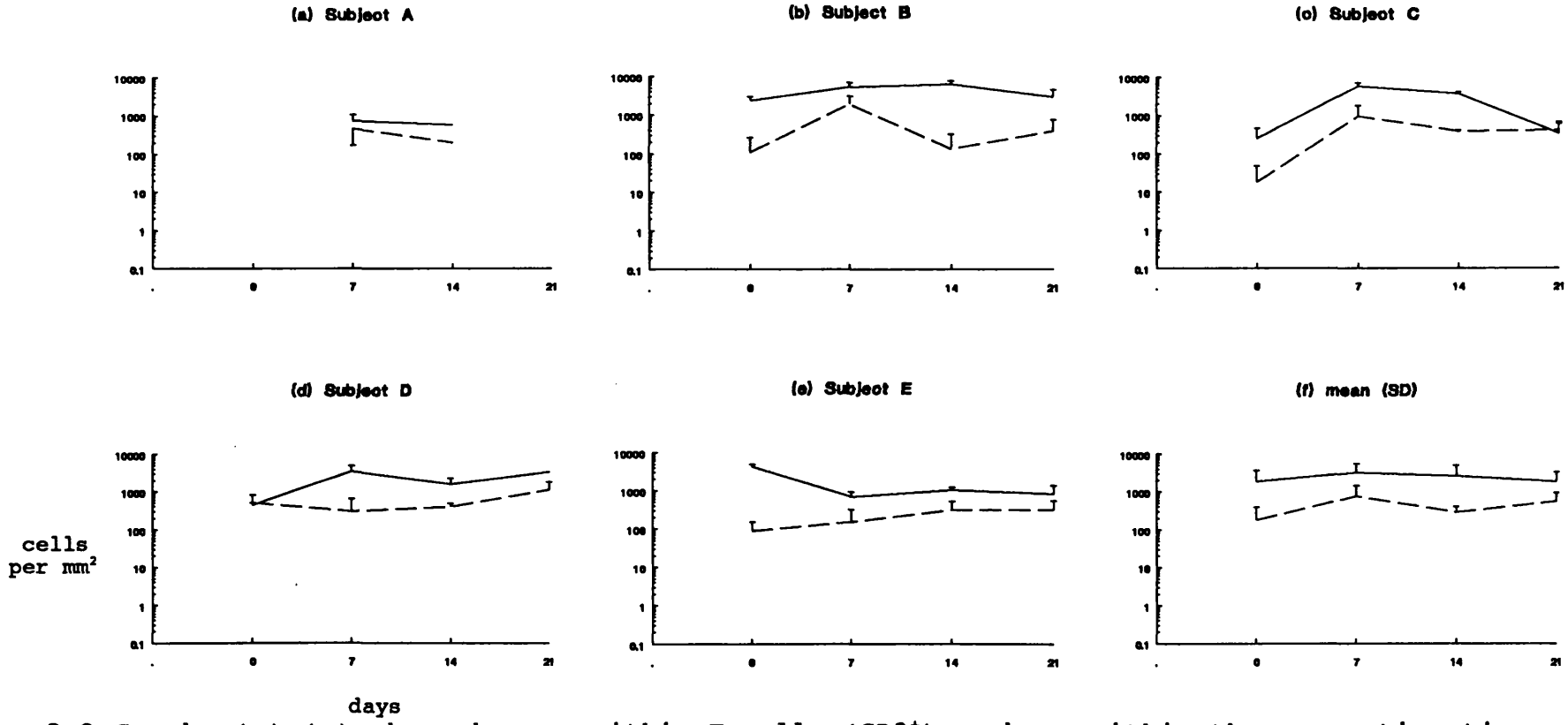

Figure 3.9 Graphs (a)-(e) show changes within T-cells ($CD3^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

	N	MEDIAN	Q1	Q3	95% CONFIDENCE INTERVAL		N	MEDIAN	Q1	Q3	95% CONFIDENCE INTERVAL
JE/SE D0	4	100	25	410	(-170.4, 526.9)	OE D0	4	15	1	62	(-28.1, 80.1)
JE/SE D7	5	210	109	2008	(-804.2,2582.2)	OE D7	5	160	110	251	(71.2, 281.2)
JE/SE D14	5	855	340	1709	(68.5,1911.9)	OE D14	5	86	53	173	(25.3, 189.9)
JE/SE D21	4	958	653	1322	(425.8,1529.7)	OE D21	4	35	22	121	(-35.5, 153.5)

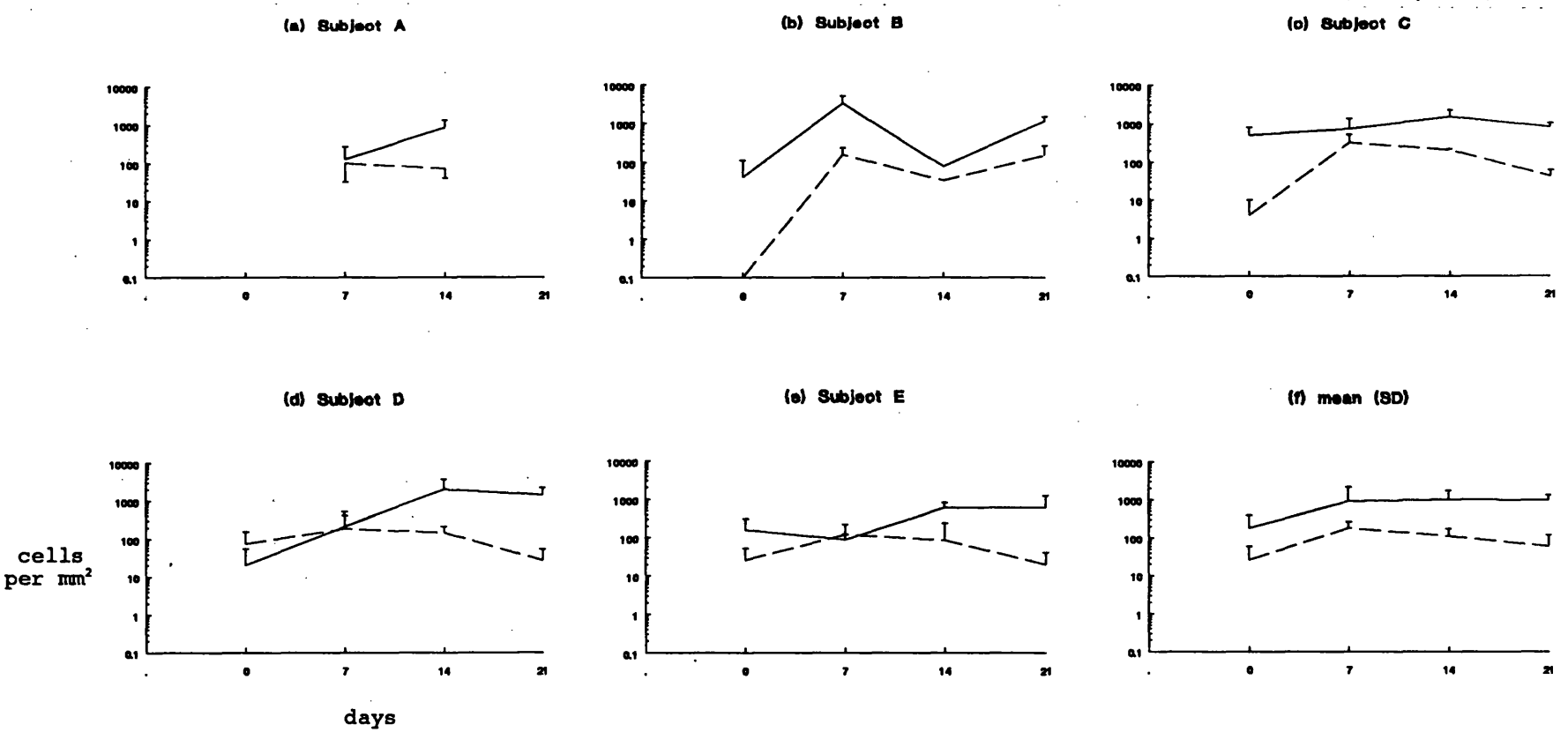

Figure 3.10 Graphs (a)-(e) show changes within T-cell ($CD3^+$) numbers within the junctional epithelium/sulcular epithelium (JE/SE) (——) and oral epithelium (OE) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

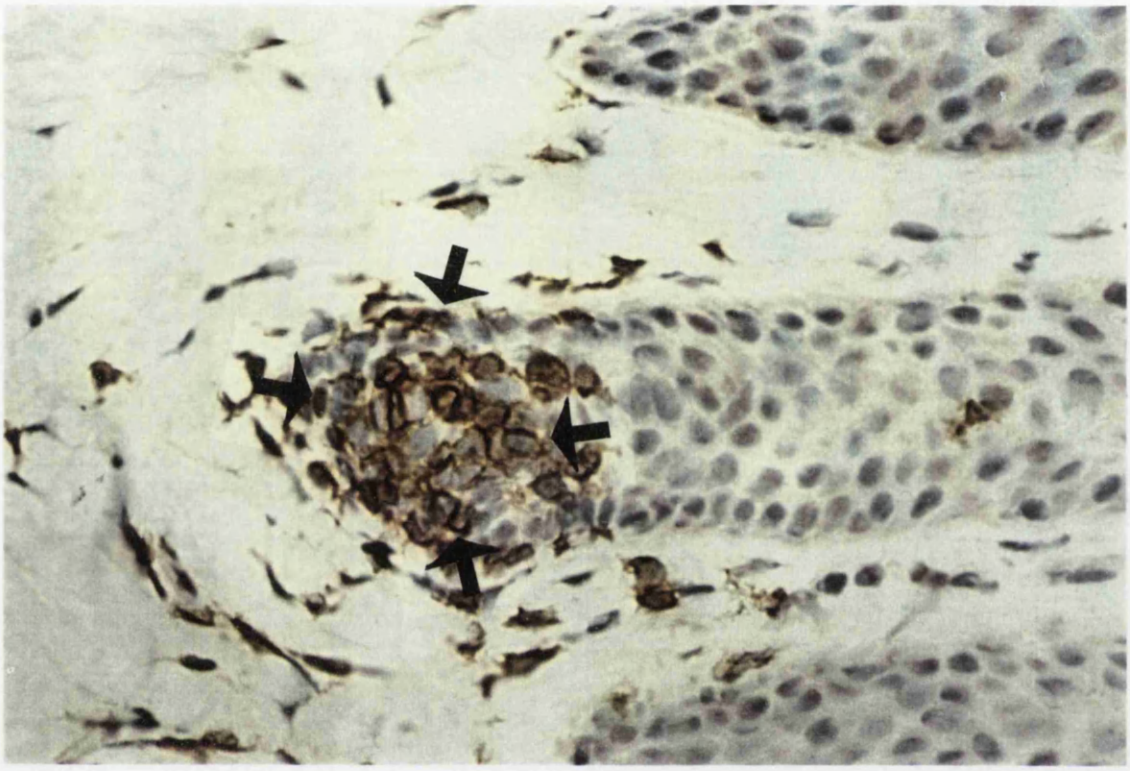

Figure 3.11 Day 14 biopsy showing T-cells ($CD3^+$) clustered around the oral epithelium rete peg tip (solid arrows) (Immunoperoxidase, mag. x500).

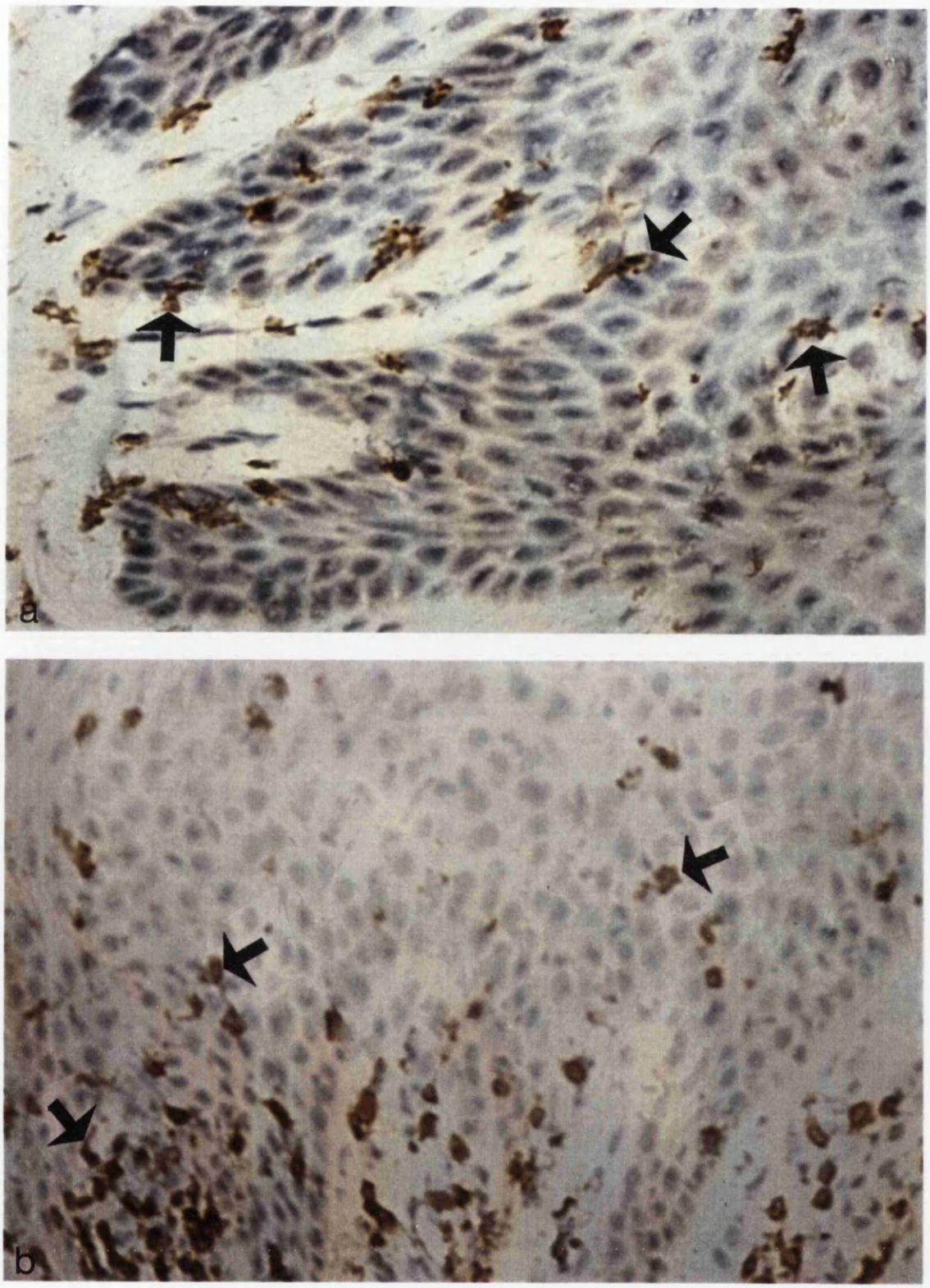

Figure 3.12 (a) $CD3^+$ T-cells located within the basal layer, stratum spinosum and stratum granulosum layer of the oral epithelium (solid arrows); **(b)** Intraepithelial lymphocytes ($CD3^+$ T-cells) seen within the junctional epithelium (Immunoperoxidase, mag. x500).

increased between day 0 and day 7 and decreased between day 7 and day 21 ($p=0.041$ and $p=0.044$, respectively). Subject also had a significant effect on $CD3^+$ cell accumulation ($p<0.02$) (MANOVA, SPSS).

With the exception of the OE where a few T cells but no PMNs were present, there was no significant difference in the numbers of T cells compared to the numbers of PMNs in the different tissue compartments at day 0. However, by day 7 as inflammation developed, there were significantly more T cells than PMNs present in each of the tissue compartments ($p<0.05$ in each case) (Mann-Whitney, Minitab) although by day 14 and day 21 these differences were still only significant in the OE and OE/CT (Table 3.6).

3.1.1.6 T cell subsets - T helper cells

Generally it was found that $CD4^+$ cells were located in all four areas (JE/SE, OE, JE/CT and OE/CT) (Figs. 3.13 & 3.14), with the numbers of cells in JE/CT higher than the numbers of cells within OE/CT (individual subject basis). These differences were only significantly elevated after 7 days of plaque accumulation i.e. when no $CD4^+$ cells were seen within OE/CT ($p<0.001$, Mann-Whitney, SPSS).

$CD4^+$ cells within the connective tissue were seen around the OE rete pegs and some positive cells were also clustered around the tips of the rete pegs (similar to $CD3^+$ cells). IELs were present in some of the biopsies located

Table 3.6 PMN:T ratios during 21-days of experimentally induced gingival inflammation.

Day	Ratios	T cells and PMNs compared in			
	p value	JE/CT	OE/CT	JE/SE	OE
0	PMN:T*	1:0.7	1:4	1:1	0:28
	p value	NS	NS	NS	<0.001
7	PMN:T	1:7	1:37	1:15	0:193
	p value	<0.05	<0.005	<0.05	<0.001
14	PMN:T	1:3	0:285	ND	0:108
	p value	NS	<0.001		<0.001
21	PMN:T	1:2	1:15	1:1	0:59
	p value	NS	<0.05	NS	<0.001

ND: Not Done; NS: Not Significant

* ratios calculated using the average number of PMNs and T-cells

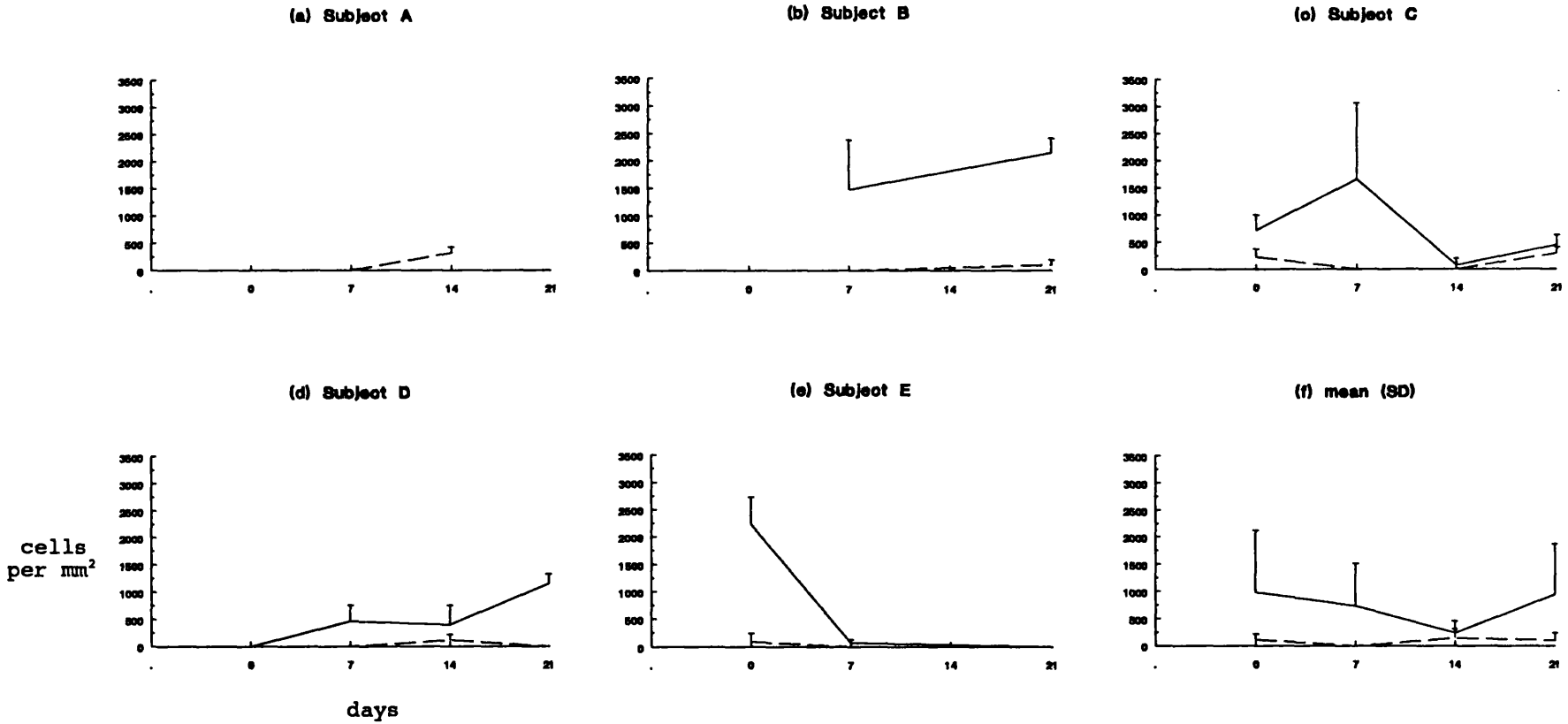

Figure 3.13 Graphs (a)-(e) show changes within helper T-cells ($CD4^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

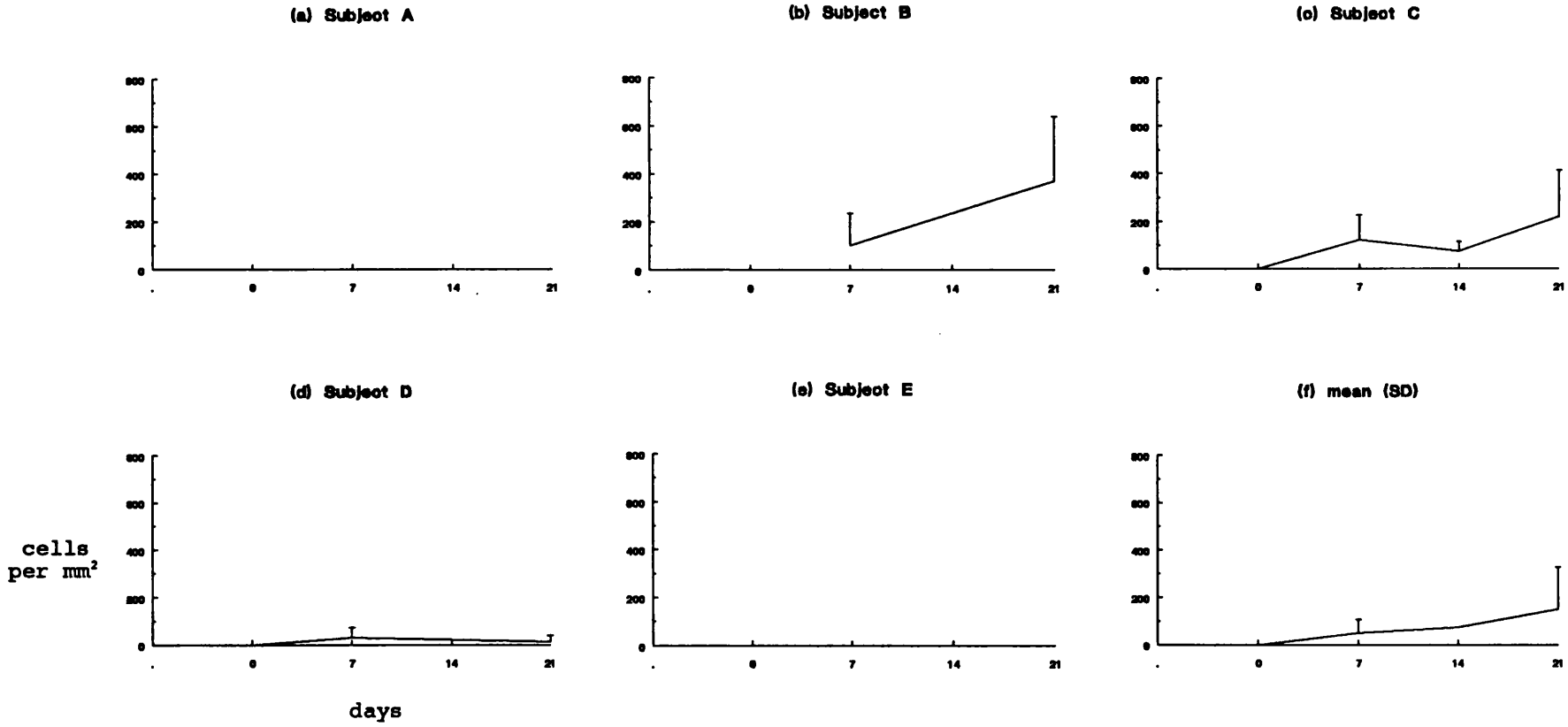

Figure 3.14 Graphs (a)-(e) show changes within helper T-cell ($CD4^+$) numbers within the junctional epithelium/sulcular epithelium (JE/SE) (——) and oral epithelium (OE) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

within the basal, stratum spinosum and stratum granulosum layers of the OE. Overall JE/CT contained more $CD4^+$ cells than the OE/CT, OE/CT had more than JE/SE and JE/SE had more than OE on an individual subject basis.

MANOVA was not performed on the CD4 data as only two full data sets were available.

3.1.1.7 T cell subsets - T cytotoxic/suppressor cells

All 4 tissue areas (JE/SE, OE, JE/CT and OE/CT) of the gingival biopsies examined contained $CD8^+$ cells (Figs. 3.15 & 3.16), although differences in cell numbers were not statistically significant with time when analysed by MANOVA (SPSS, n=3 subjects B, C, D).

Cells appeared to be located in higher numbers in JE/CT than OE/CT (on an individual subject basis), although differences were not statistically significant (Mann-Whitney, Minitab). IELs were also present and seen in all biopsies, unlike $CD4^+$ cells. The $CD8^+$ IELs were located in basal and stratum spinosum layers of the OE but as inflammation developed (day 7), IELs were noted within the stratum granulosum layer, and by day 14 and day 21 the cells were located again at the basal layer. Positive cells were also detected within connective tissue areas with some cells tending to cluster around the tip of the OE rete pegs as seen with the $CD3^+$ and $CD4^+$ cells.

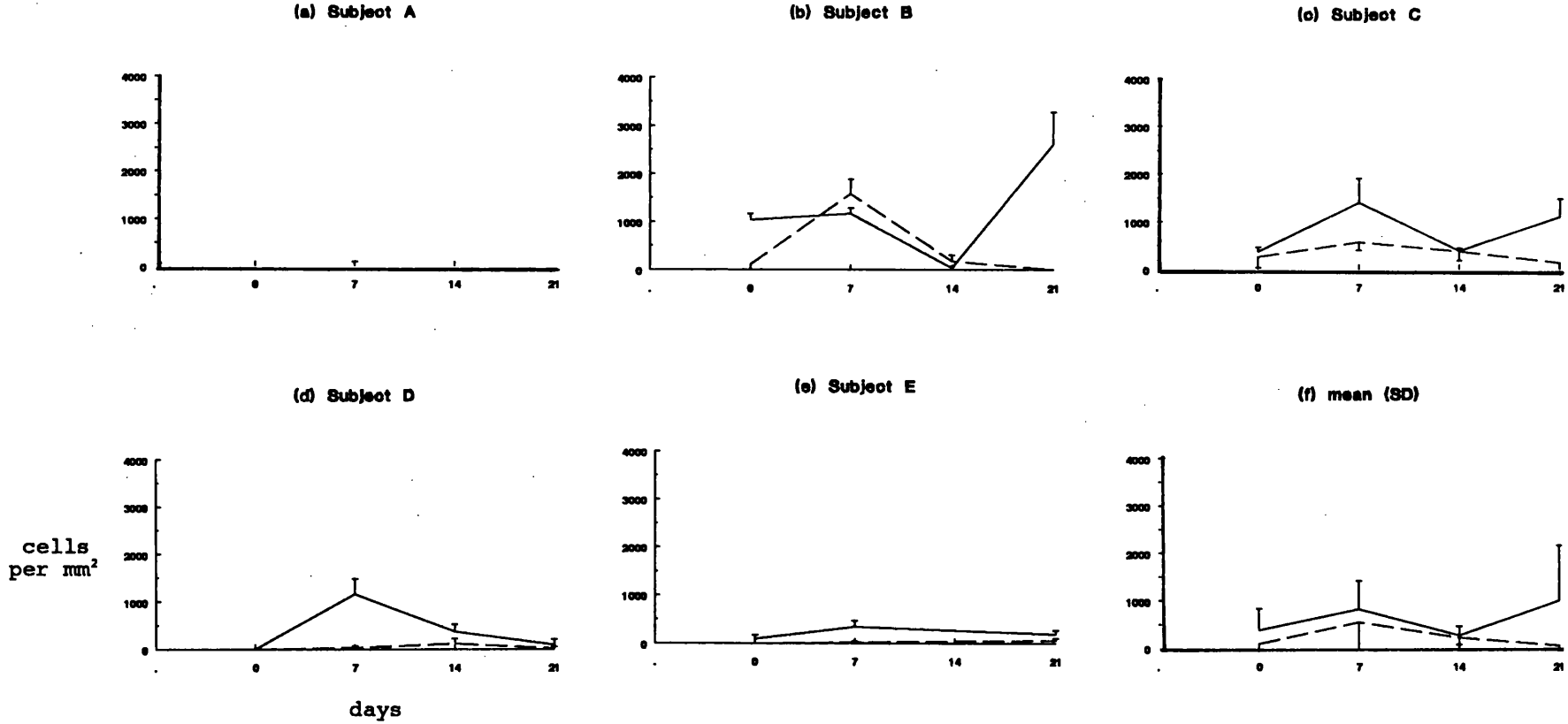

Figure 3.15 Graphs (a)-(e) show changes within cytotoxic/suppressor T-cells ($CD8^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

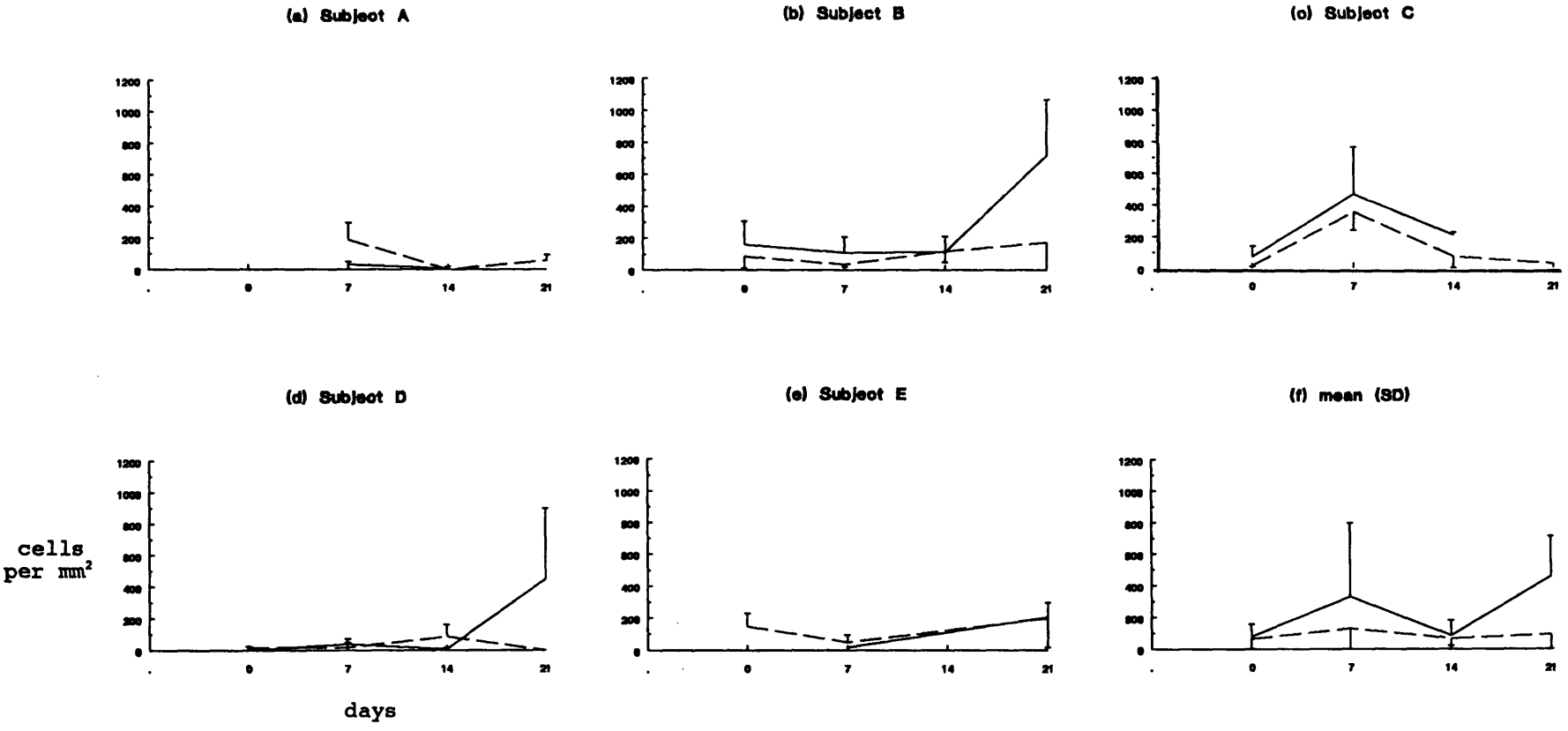

Figure 3.16 Graphs (a)-(e) show changes within cytotoxic/suppressor T-cell ($CD8^+$) numbers within the junctional epithelium/sulcular epithelium (JE/SE) (——) and oral epithelium (OE) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

The number of T helper to T cytotoxic/suppressor cells within JE/CT and OE/CT at different time points were compared and it was found that the T4:T8 ratio did not vary significantly, except at day 7, when within OE/CT there were no detectable $CD4^+$ cells.

3.1.1.8 T cell subsets - T memory cells

Cells positive for the memory marker ($UCHL\text{-}1^+$) were found within all biopsies at all time points (Figs. 3.17 & 3.18). Changes in memory T cell number were not significant when analysed over time or by subject (MANOVA, SPSS).

Generally $UCHL\text{-}1^+$ cells were seen more within JE/CT than OE/CT, although differences were only significant at day 7, where T memory cells in JE/CT were higher in number ($p=0.0048$) (Mann-Whitney, Minitab). IELs when present within OE, were located within the basal and stratum spinosum layer and only one day 7 biopsy had IEL within the stratum granulosum layer. Positive cells within connective tissue were seen around the OE rete peg tips (Fig. 3.19). Overall positive cells were located within JE/CT with lower numbers of T memory cells within OE/CT, JE/SE and OE.

3.1.1.9 Langerhans cells

At day 0 in healthy gingiva, $CD1a^+$ LCs were seen within the oral and sulcular epithelia. Generally they were dendritic and were found within the stratum spinosum of the gingival OE, with only a few cells present within the sulcular

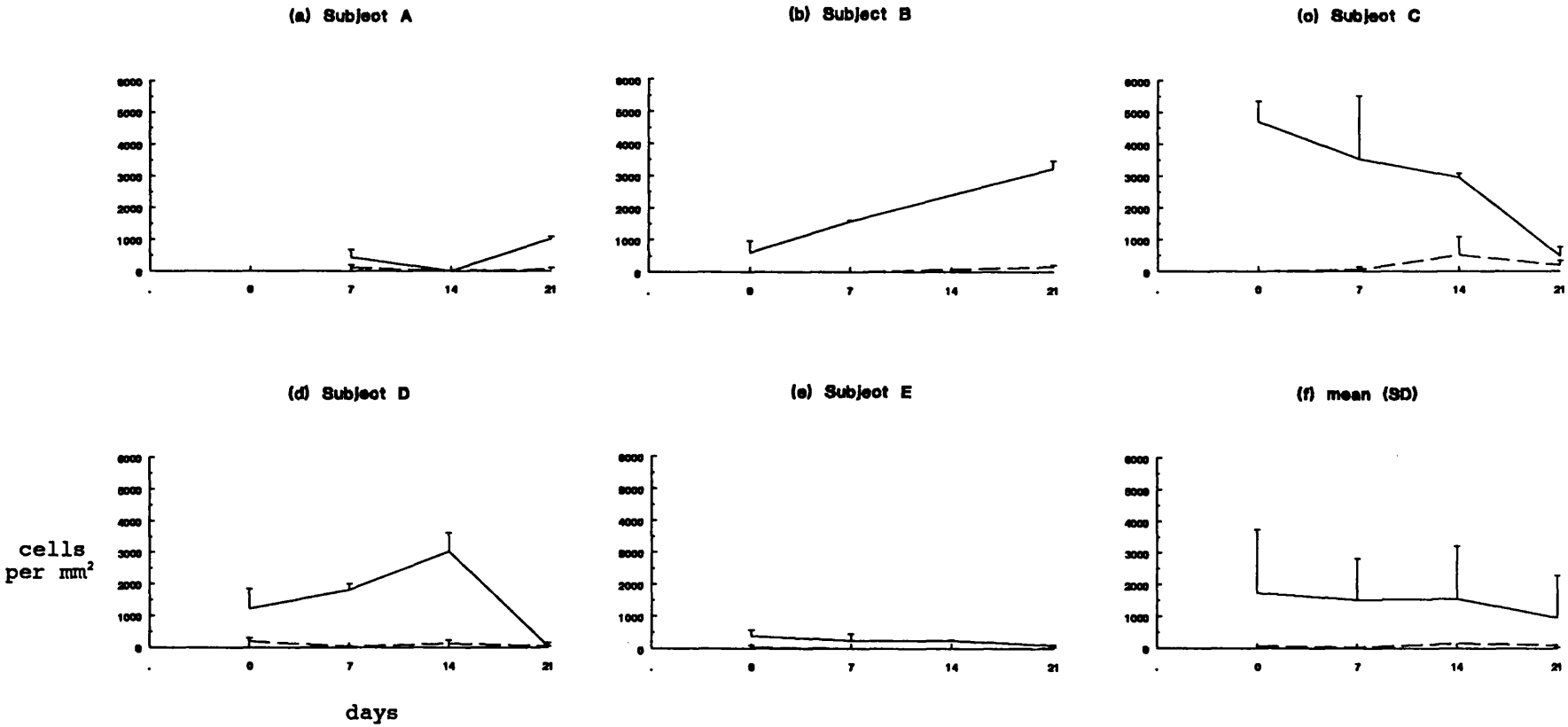

Figure 3.17 Graphs (a)-(e) show changes within memory T-cells (UCHL-1$^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

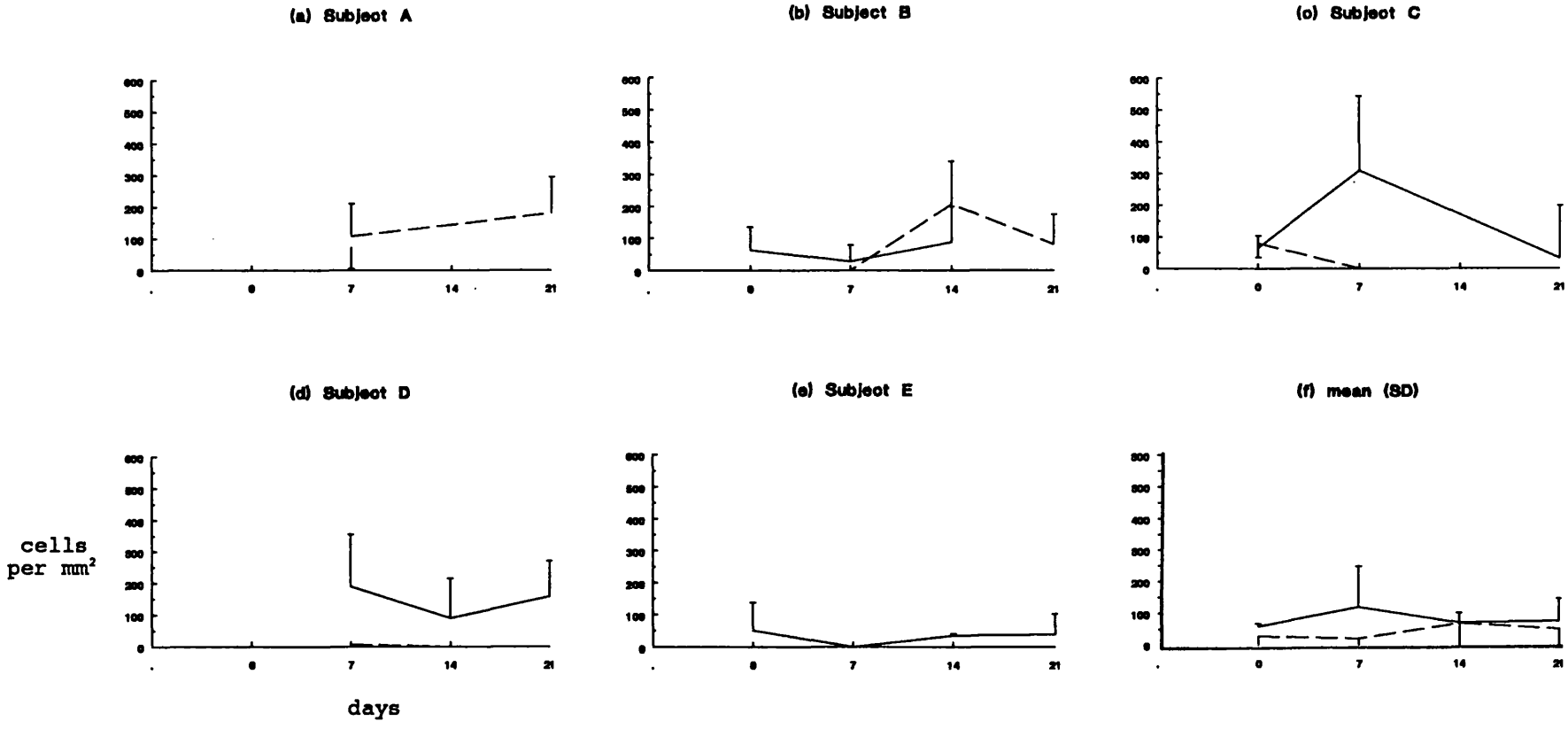

Figure 3.18 Graphs (a)-(e) show changes within memory T-cell (UCHL-1$^+$) numbers within the junctional epithelium/sulcular epithelium (JE/SE) (——) and oral epithelium (OE) (---) for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

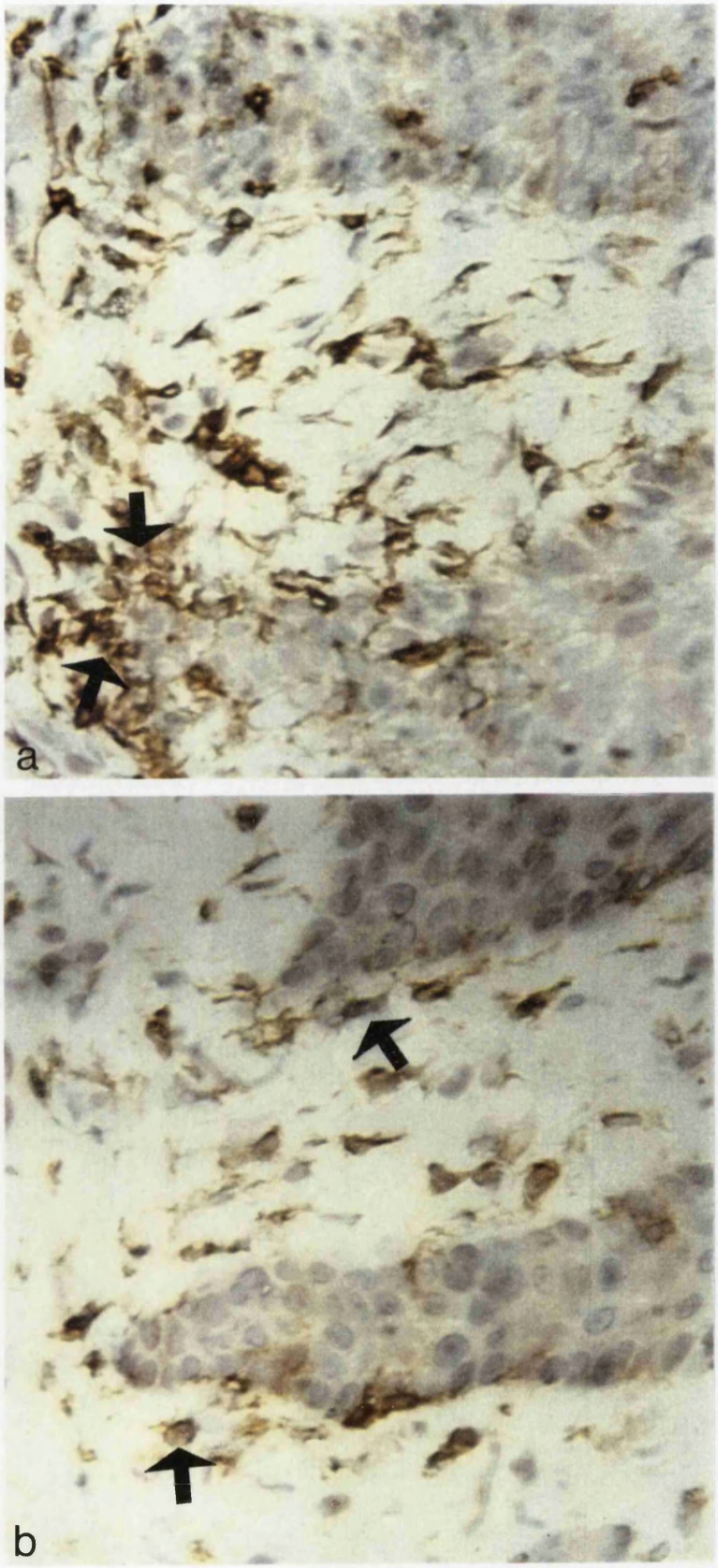

Figure 3.19 (a) Day 0 **(b)** Day 14 biopsy showing memory T-cells (UCHL-1^{+}) clustered around the oral epithelium rete peg tips (solid arrows) (Immunoperoxidase, mag. x400).

epithelium. Junctional epithelium was often incomplete and variable and the term sulcular epithelium was used to include both junctional and oral sulcular epithelium. As plaque accumulated there was a shift of LCs from the stratum spinosum to the stratum granulosum (day 0 to day 7) and by day 14 the $CD1a^+$ LCs were more basally positioned (Fig. 3.20). $HLA\text{-}DR^+$ dendritic cells were found within the gingival OE and were morphologically identical to the $CD1a^+$ LCs (Fig. 3.21), strongly suggesting that the two monoclonal antibodies detected the same cell type within the gingival OE. HLA-DR antigens were detected on 50-85% of $CD1^+$ LCs (calculated from serial sections). Keratinocytes only faintly stained for HLA-DR and combined with their morphological appearance permitted clear distinction from LCs to be made.

As the clinical indices increased, the density of $CD1a^+$ LCs within the gingival OE increased slightly during the first seven days of oral hygiene abstention. Following this initial increase, $CD1a^+$ LC numbers decreased with further plaque accumulation and development of gingival inflammation (day 7 to day 14). However, as inflammation developed, the numbers of $CD1a^+$ LCs detected within the gingival OE decreased back to baseline levels by day 21. The number of $HLA\text{-}DR^+$ LCs however increased and plateaued between day 7 and day 14, then decreased to baseline levels by day 21 (Fig. 3.22).

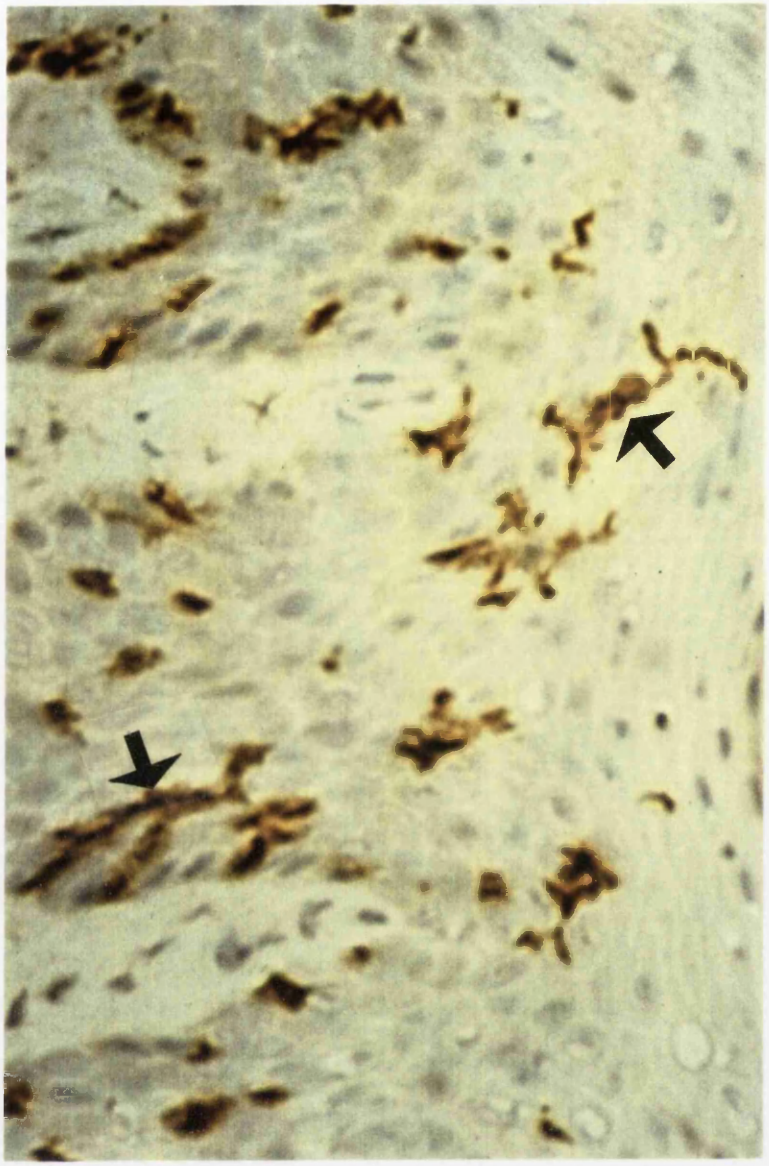

Figure 3.20 Day 7 biopsy with $CD1^+$ Langerhans cells within the oral epithelium (solid arrows) (Immunoperoxidase, mag. x500).

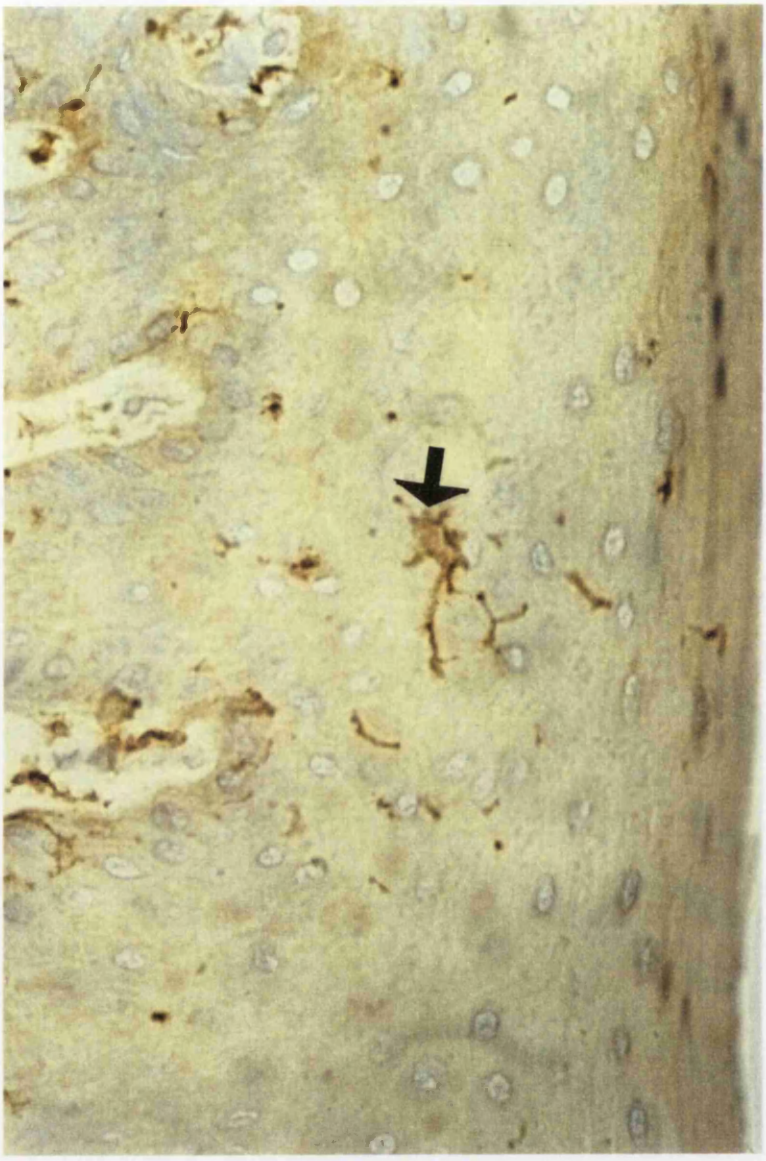

Figure 3.21 Day 14 biopsy: A HLA-DR^{+} Langerhans cell within the oral epithelium (solid arrow) (Immunoperoxidase, mag. x500).

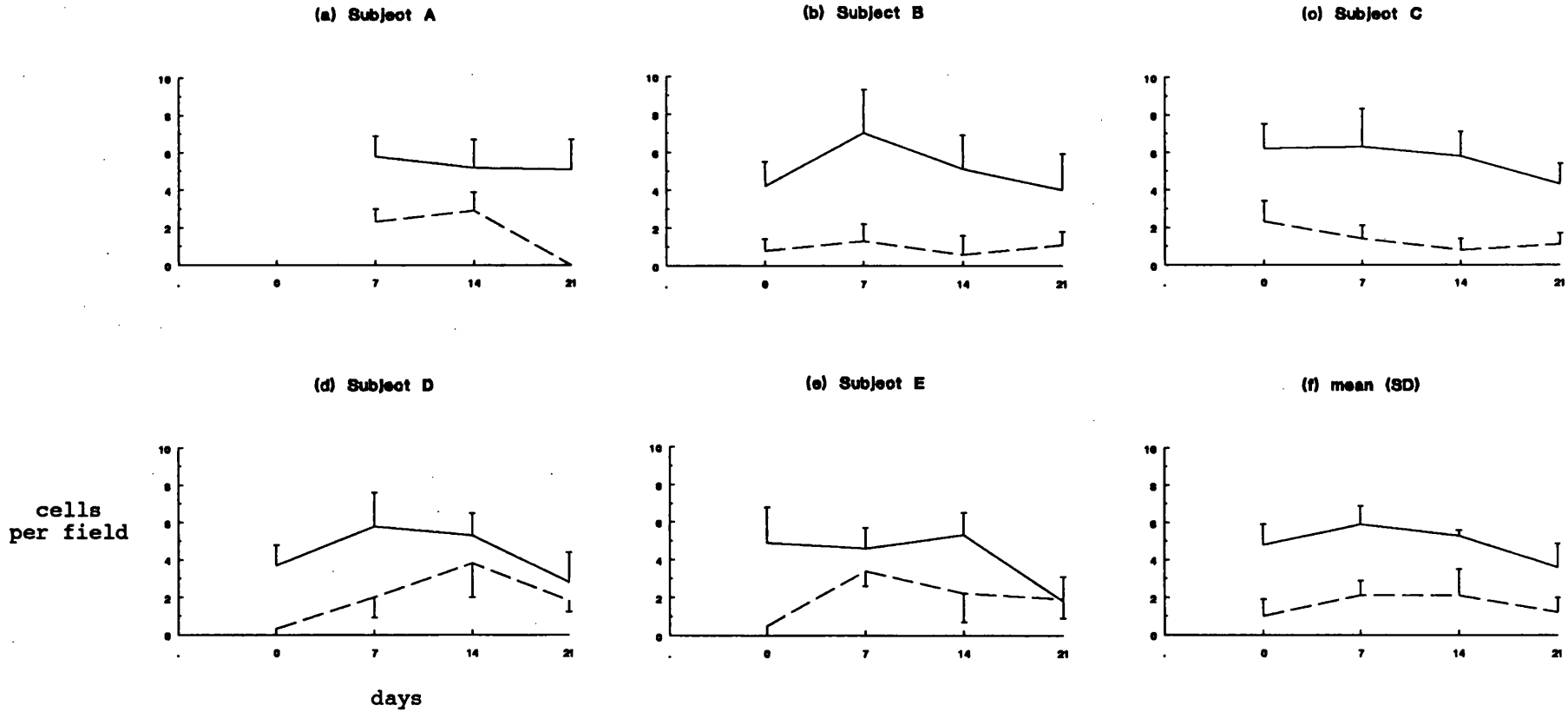

Figure 3.22 Graphs (a)-(e) show changes within Langerhans cells numbers, CD1a$^+$ (——) and HLA-DR$^+$ (---), within the oral epithelium for subjects A-E respectively, during the 21-day experimental gingivitis study. Each point represents the mean and standard deviation of 10 fields. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

MANOVA (SPSS) demonstrated significant changes in the numbers of $CD1a^+$ LCs with time ($p=0.005$) and further analysis (paired t-tests, SPSS) demonstrated that the decrease in $CD1a^+$ LCs which occurred between day 7 and 21 and between day 14 and 21 were statistically significant ($p=0.003$ and $p=0.028$ respectively). However, no statistically significance was demonstrated when MANOVA was used to analyses $HLA\text{-}DR^+$ LCs changes. Subject had a significant effect on the levels of expression of HLA-DR and CD1a ($p=0.008$ and $p=0.001$ respectively). These p levels are still statistically significant despite correction for the multiple comparisons (n=6) which reduces the statistically significant threshold to 0.02.

3.1.1.10 Interleukin-1

GCF was collected mid-buccally from the first permanent molars (section 2.5.1), prior to biopsing the sites. Four GCF strips were collected from each subject (one at each time point, n=24 strips). IL-1 levels and GCF volume variations for the six subjects are shown in Figures 3.23 and 3.24 respectively. As plaque accumulated (day 0 to day 21) there was an overall increase in GCF volume which was not statistically significant when analysed by MANOVA (SPSS), although subject had a significant effect ($p=0.003$) (MANOVA, SPSS).

The IL-1 concentration (ng/μl) increased with plaque accumulation and in advance of subsequent gingival

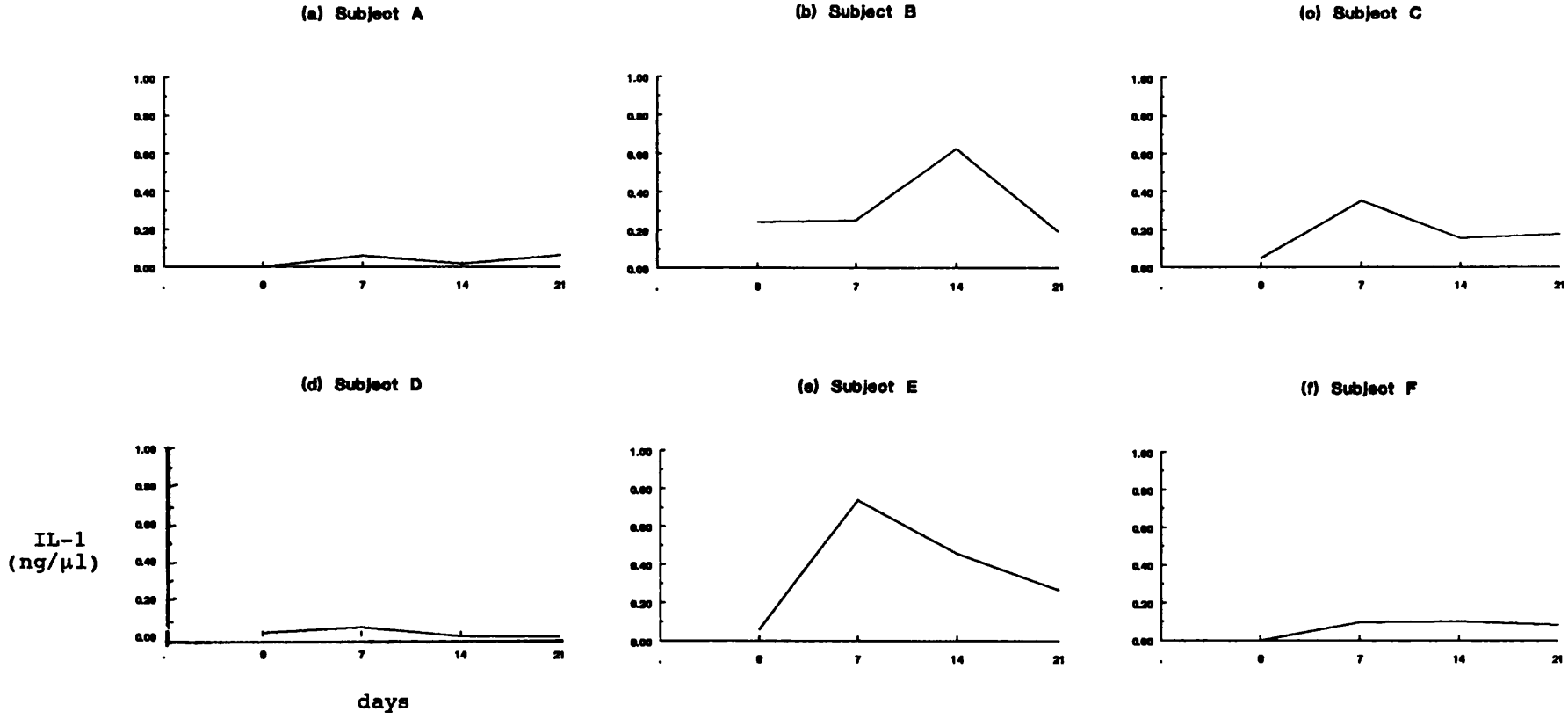

Figure 3.23 Graphs (a)-(f) show changes in interleukin-1 (IL-1) concentration within gingival crevicular fluid samples collected from the mid-buccal area of the biopsy sites for subjects A-F respectively, during the 21-day experimental gingivitis study. The x-axis and y-axis legends for all the graphs are identical.

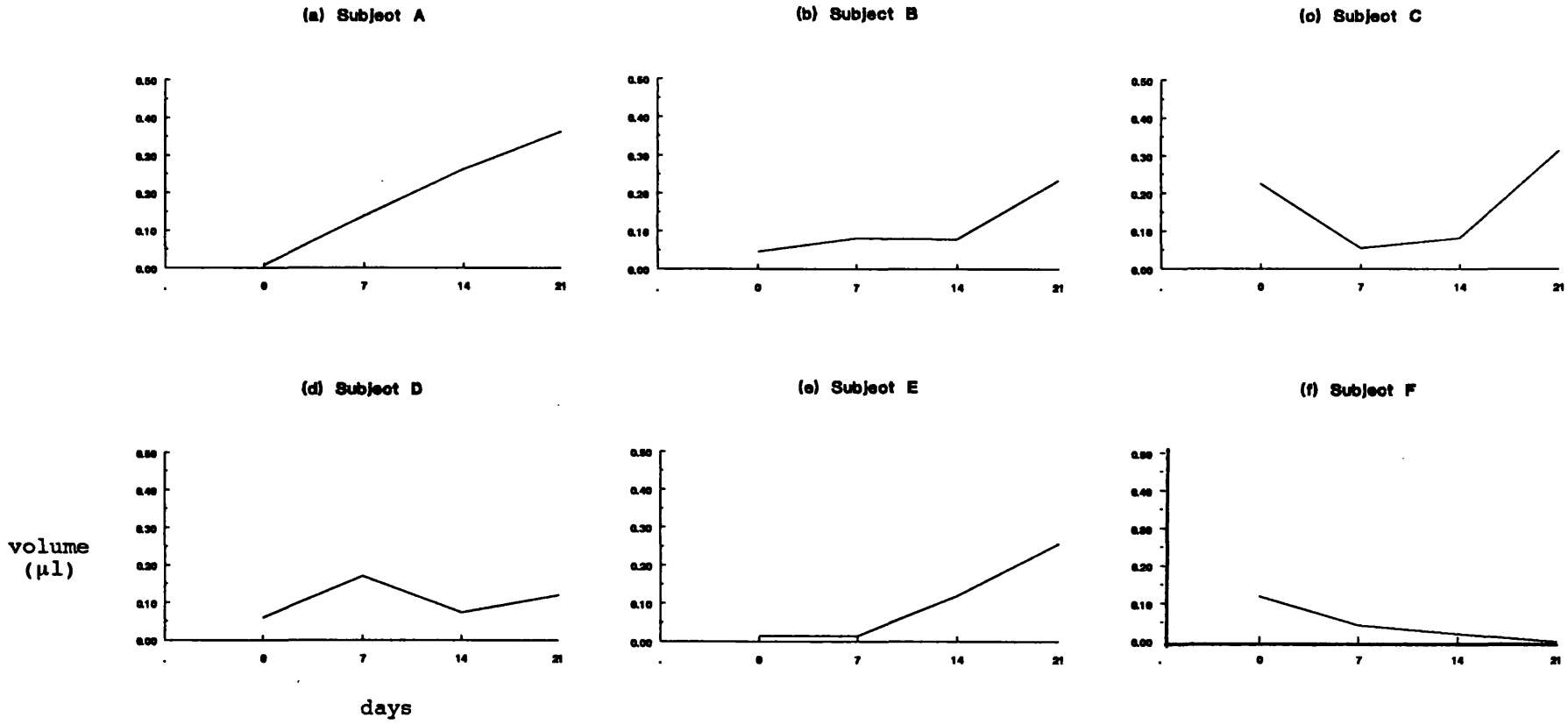

Figure 3.24 Graphs (a)-(f) show changes in gingival crevicular fluid (GCF) volume from GCF samples collected from the mid-buccal area of the biopsy sites for subjects A-F respectively, during the 21-day experimental gingivitis study. The x-axis and y-axis legends for all the graphs are identical.

inflammation. The average IL-1 level for the 21 day experimental gingivitis group began to drop well before normal oral hygiene was reinstituted and returned to baseline levels ahead of the inflammation index (MGI) though not statistically significant (MANOVA, SPSS) (Fig. 3.25).

Pearson's correlation coefficient and regression analysis was performed on the mean IL-1 levels, mid-PI and mid-MGI for each subject summed over the four sampling intervals (Table 3.7). Mid-PI and IL-1 levels were both positively correlated with the mid-MGI, although the relationship between mid-PI and IL-1 was less strong. There was intersubject variation in the quantity of crevicular IL-1 ($p=0.046$, $n=6$ subjects) (MANOVA, SPSS). Two of the subjects developed a rise in crevicular IL-1 activity which peaked within 7 days of withdrawing oral hygiene, 3 demonstrated minimal crevicular IL-1 activity and the remaining subjects crevicular IL-1 activity peaked at day 14 (Fig. 3.23).

3.1.2 Ten-day experimental gingivitis study

Five individuals (G-K) participated and completed this study (section 2.2.1). Clinical indices, GCW samples and gingival biopsies were collected and processed as previously mentioned.

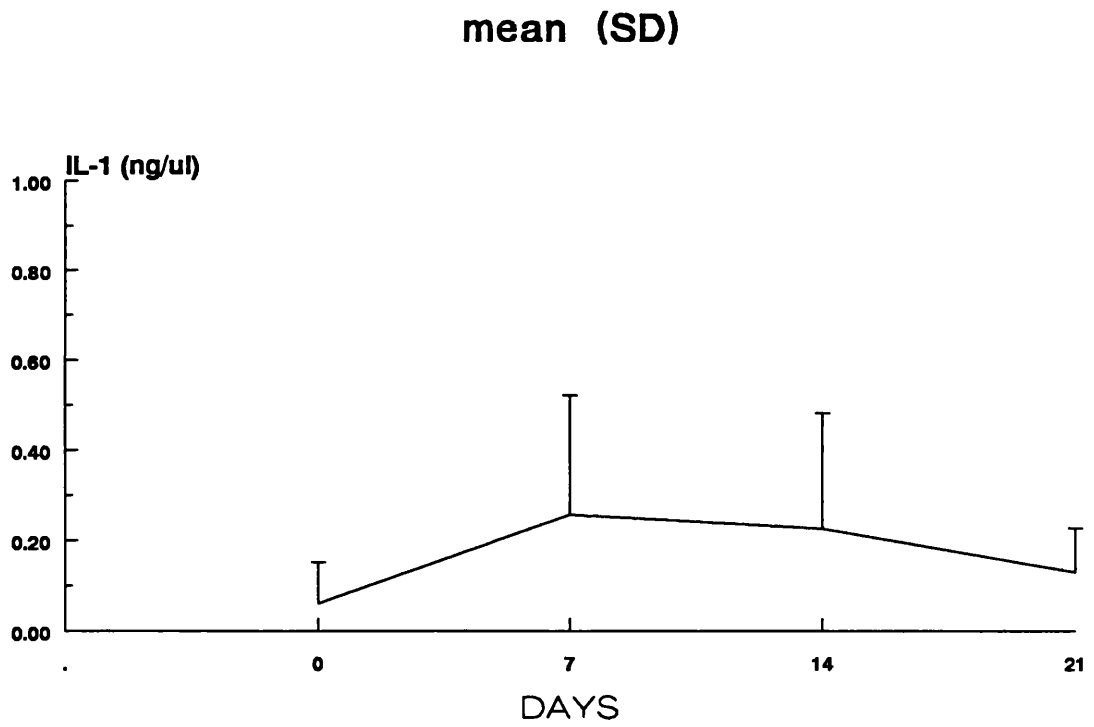

Figure 3.25 Shows the change in the interleukin-1 (IL-1) concentration mean for the individual subject means (n=6), during the 21-day experimental gingivitis study.

Table 3.7 Regression analysis of clinical indices and crevicular IL-1 (ng/μl). Pearson's correlation coefficient (r) and probability values (p) are shown. Samples collected from mid-buccal aspect of biopsy sites.

Parameters	r	p
PI vs IL-1 (ng/μl)	0.667	NS
PI vs MGI	0.765	0.077
MGI vs IL-1 (ng/μl)	0.860	0.028

MGI: Modified Gingival Index; NS: Not Significant;
PI: Plaque Index

3.1.2.1 Clinical indices

Clinical indices were collected every 3 days of the 10 day experimental phase. PI (Silness & Löe, 1964) and MGI (Lobene *et al.*, 1986) were scored for the mesial and mid-buccal aspect of the first permanent molars prior to GCW collection and subsequent biopsing.

In clinically 'healthy' gingiva (day 0) all subjects had minimal plaque (PI=0) and no clinically visible gingival inflammation (MGI=0) mid-buccally (Fig. 3.26) or mesially (Fig. 3.27).

At the mid-buccal aspect plaque accumulated at different rates within the different individuals, i.e. Subject I had no plaque at all whereas subject G showed continuous accumulation, though when analysed with Friedman's test (section 2.7.7.2) these subject and time effects were not significant (Friedman's test, Minitab). Gingival inflammation within the individual also developed at different rates, 3 subjects had MGI scores of 0 at day 7, one subject developed visible inflammation by day 3 and the remaining subject developed visible gingival inflammation after 7 days of oral hygiene abstention.

Mesially the clinical indices increased with time with the mesial-PI rise preceding the rise in mesial-MGI (Fig. 3.27). There was large subject variation though this did not reach statistical significance (Friedman's test,

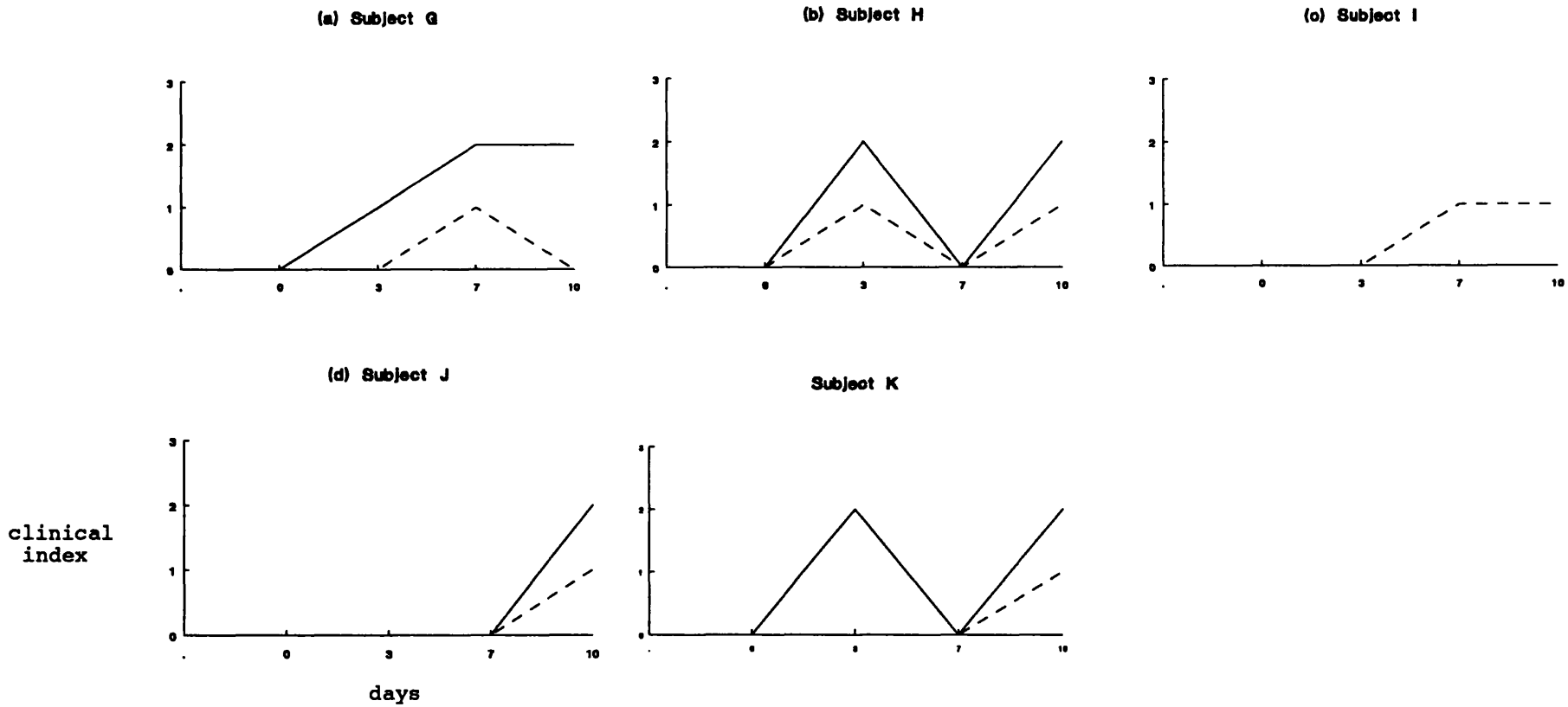

Figure 3.26 Clinical index scores for the mid-buccal area (i.e. the biopsy site) obtained during the 10-day experimental gingivitis study. Graphs (a)-(e) show individual site scores for the plaque index (PI) (——) and the modified gingival index (MGI) (---) for subjects G-K respectively. The x-axis and y-axis legends for all the graphs are identical.

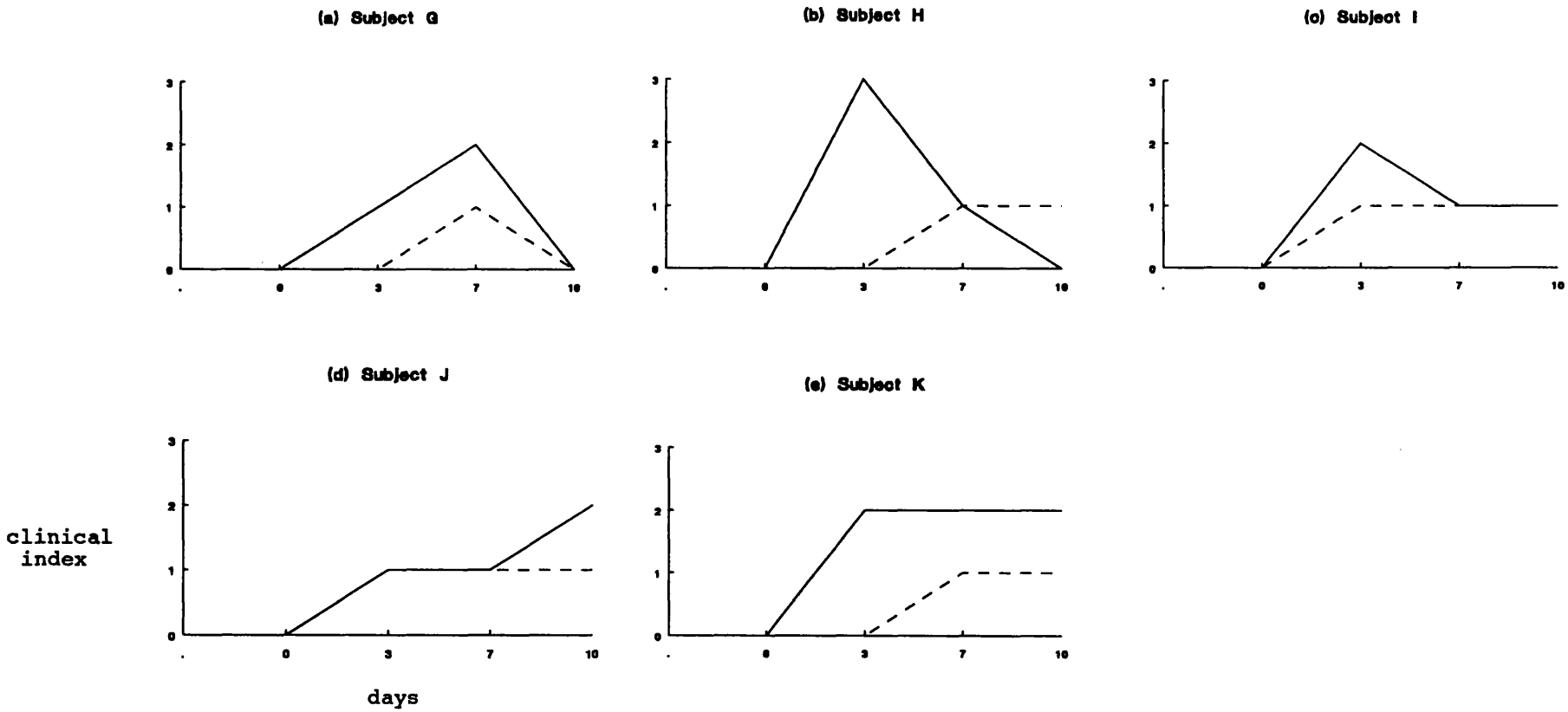

Figure 3.27 Clinical index scores for the mesial area (i.e. the gingival crevicular washing site) obtained during the 10-day experimental gingivitis study. Graphs (a)-(e) show individual site scores for the plaque index (PI) (——) and the modified gingival index (MGI) (---) for subjects G-K respectively. The x-axis and y-axis legends for all the graphs are identical.

Minitab). The mesial aspect when analysed for the effect of day on the indices both mesial-PI and mesial-MGI increased significantly the longer oral hygiene was abandoned (p=0.027 and p=0.016 respectively, Friedman's Test, Minitab).

Associations between clinical indices and days oral hygiene abstention were analysed using the non-parametric Spearman's rank correlation coefficient and bi-variate regression analysis. Mid-PI, mid-MGI and mesial-MGI demonstrated positive correlations with time (Table 3.8).

3.1.2.2 Haematoxylin and eosin sections

Clinically 'healthy' gingival tissue exhibited a wide variation in the extent of cellular infiltration. Some subjects exhibited more cellular infiltrate than others (subject G compared with subject H). During the development of inflammation (day 3 to day 10) changes in the size of cellular infiltration were seen but were not consistent (Table 3.9). No correlation between the size of cellular infiltration and days of plaque accumulation or mid-MGI was seen even though a strong correlation existed with mid-PI (r=0.525, p=0.017) (Spearman's rank correlation coefficient and regression analysis, Minitab) (Table 3.10).

Histologically the clinically 'healthy' gingival tissue (day 0) was well structured, but as inflammation developed their was a slight loss of tissue integrity; rete peg

Table 3.8 Regression analysis of clinical indices obtained during the 10-day experimental gingivitis study. Spearman rank correlation coefficient (r) and probability values (p) are shown.

Parameters	r	p
Mid-PI vs Day	0.465	0.039
Mid-MGI vs Day	0.606	0.005
Mid-PI vs Mid-MGI	0.517	0.020
Mesial-PI vs Day	0.331	NS
Mesial-MGI vs Day	0.686	0.001
Mesial-PI vs Mesial MGI	0.430	NS

Mesial-MGI: Modified Gingival Index (mesial aspect);
Mesial-PI: Plaque Index (mesial aspect);
Mid-MGI: Modified Gingival Index (mid-buccal aspect);
Mid-PI: Plaque Index (mid-buccal aspect);
NS: Not Significant

Table 3.9 Size of cellular infiltration for each subject at each time point during the 10-day experimental gingivitis study.

Subject	Size of cellular infiltrate			
	Day 0	Day 3	Day 7	Day 10
G	+	+	++	+++
H	+++	+++	+	+
I	+	++	+	+
J	++	+	+	++
K	+	++	+	++

Table 3.10 Regression analysis of clinical indices and cellular infiltrate size during the 10-day experimental gingivitis study. Spearman's rank correlation coefficient (r) and probability values (p) are shown. Biopsies were taken from the mid-buccal aspect of the first molars.

Parameters	r	p
Day vs Infiltrate size	-0.001	NS
PI vs Infiltrate size	0.525	0.017
MGI vs Infiltrate size	0.142	NS

MGI: Modified Gingival Index; NS: Not Significant;
PI: Plaque Index

proliferation and increased vascularisation as noted by the appearance of capillaries within the JE/SE and JE areas.

3.1.2.3 Gingival crevicular PMNs

Prior to biopysing, gingival crevicular washings (GCWs) were collected from the mesial aspect of the first permanent molars and processed (section 2.6.2). In total 20 GCW were collected from subjects G-K. The percentage of PMNs found within the GCW and the calculated numbers of PMNs/μl (Figs. 3.28 & 3.29 respectively) were analysed using the Friedman's test (Minitab). The Friedman's test demonstrated no day or subject effect on the percentage of PMNs obtained or on the calculated numbers of PMNs/μl GCW, although a positive correlation existed between the mesial PI and percentage PMNs (Table 3.11).

3.1.2.4 ELAM-1, ICAM-1 and VCAM-1 expression

The 10 day study tissue was examined for expression of the same two adhesion molecules as the 21 day study (ELAM-1 and ICAM-1), as well as for VCAM-1. All three adhesion molecules demonstrated blood vessel staining within clinically 'healthy' tissue (day 0, subjects G-K) and in experimentally inflamed tissue (day 3 - 10) (Tables 3.12 - 3.14).

Staining was seen within JE/CT and OE/CT with ELAM-1 demonstrating a 20% decrease in the number of biopsies with stronger staining vessels at JE/CT when compared with OE/CT

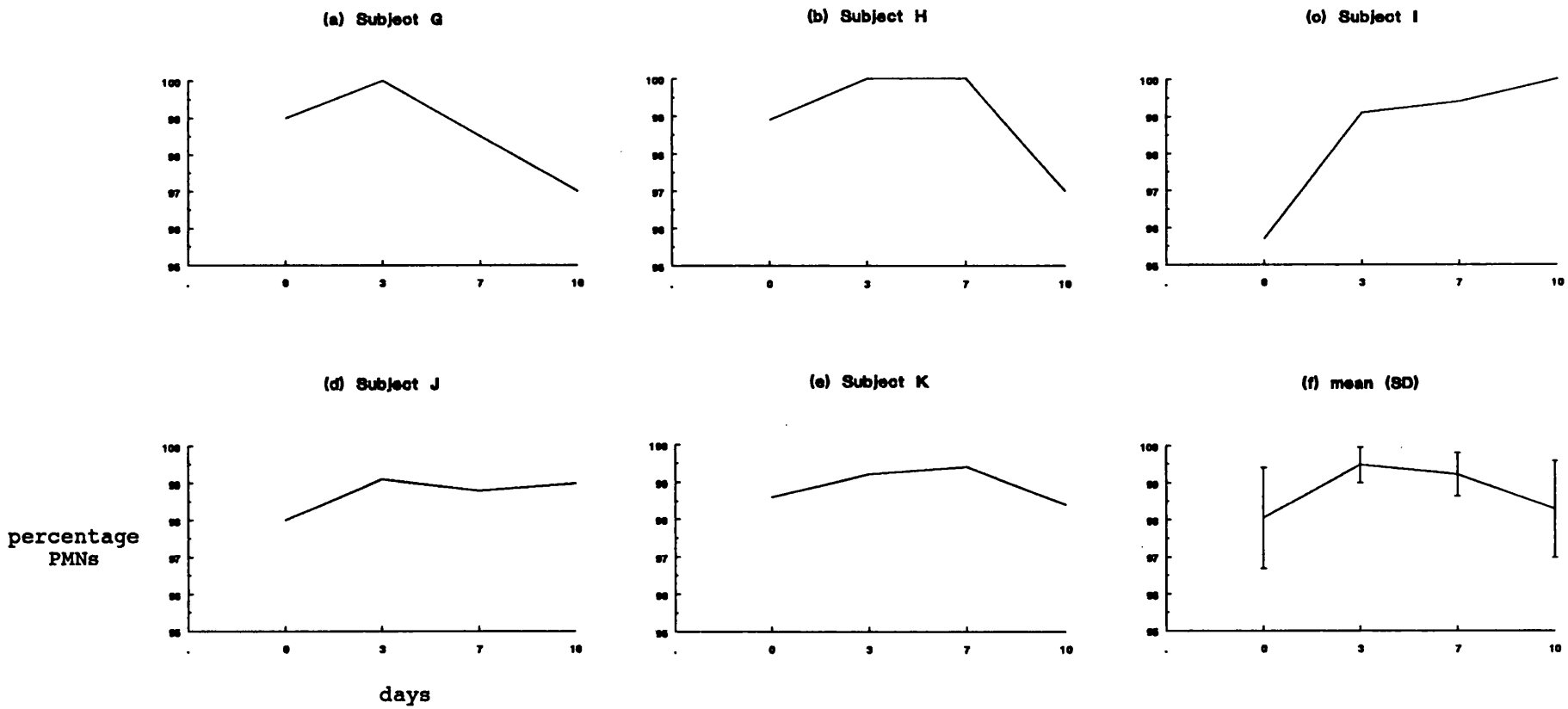

Figure 3.28 The percentage of neutrophils found within gingival crevicular washing taken from the mesial aspect of the first permanent molars prior to biopsying, during the 10-day experimental gingivitis study. Graphs (a)-(e) show changes in the percentage of neutrophils for subjects G-K respectively. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

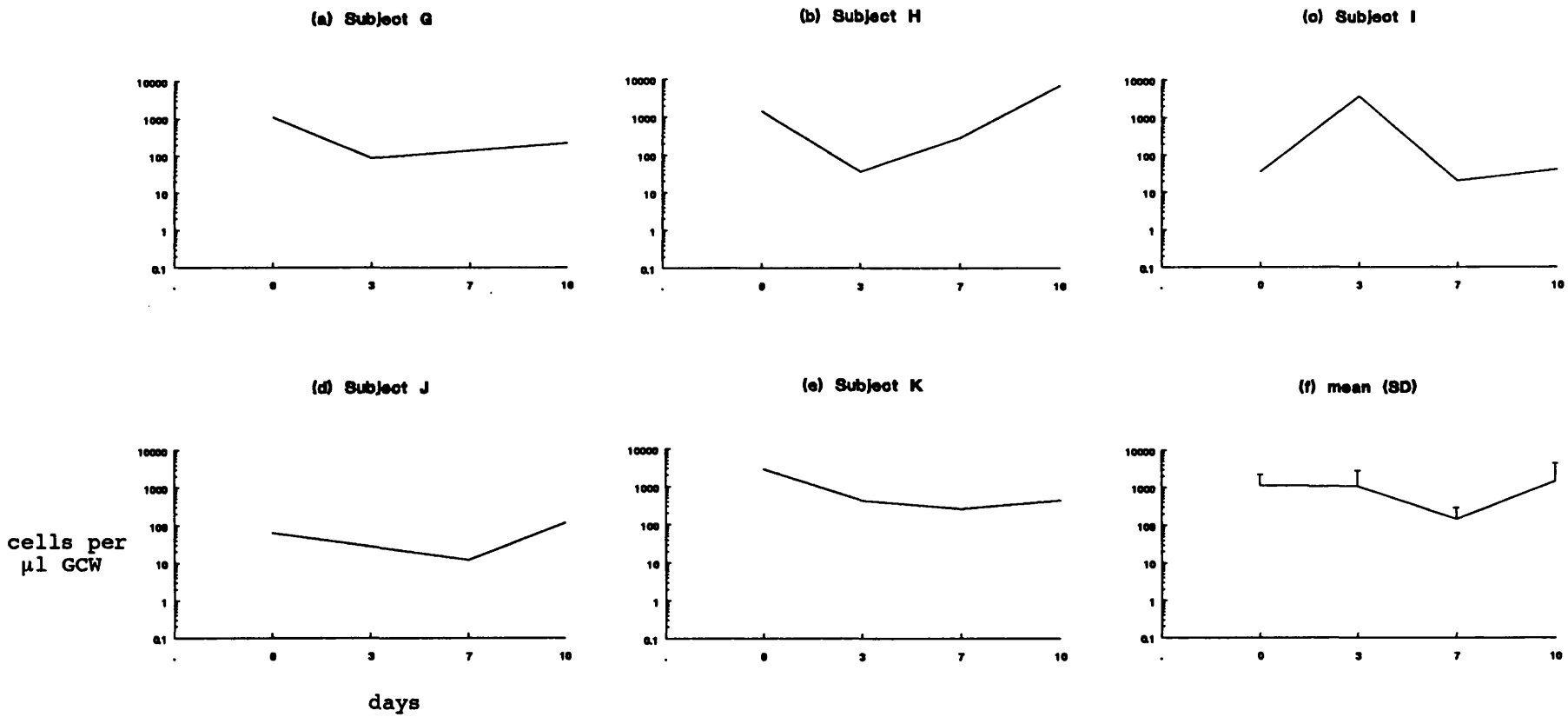

Figure 3.29 The number of neutrophils per microlitre (µl) gingival crevicular washing (GCW) is plotted against the development of 10-day gingival inflammation. Graphs (a)-(e) show changes in the numbers of neutrophils/µl GCW for subjects G-K respectively. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

Table 3.11 Regression analysis of clinical indices, percentage PMNs and PMNs/μl gingival crevicular washing during the 10-day experimental gingivitis study. Spearman's rank correlation coefficient (r) and probability values (p) are shown. The mesial aspect of the first molars were sampled.

Parameters	r	p
PI vs Percentage PMNs	0.536	0.015
PI vs PMN/μl(log^{10})	-0.196	NS
MGI vs Percentage PMNs	0.220	NS
MGI vs PMN/μl(log^{10})	-0.036	NS

MGI: Modified Gingival Index; NS: Not Significant;
PI: Plaque Index

Table 3.12 ELAM-1 staining intensity for the five subjects during the 10-day experimental gingivitis study.

Day	Subject	Intensity of ELAM-1 staining	
		JE/CT	OE/CT
0	G	++	++
	H	++/+++	++/+++
	I	++/+++	++/+++
	J	++	+
	K	+++	++
	median	++/+++	++
3	G	+/++	+/++
	H	-/+	-/+
	I	+	+
	J	+	+
	K	-/+	-/+
	median	+	+
7	G	++	+
	H	++	+
	I	++	+
	J	+	+
	K	++	++
	median	++	+
10	G	+	+
	H	++	++
	I	++	++
	J	++	+
	K	++/+++	++/+++
	median	++	++

Table 3.13 ICAM-1 staining intensity for the five subjects during the 10-day experimental gingivitis study.

Day	Subject	Intensity of ICAM-1 staining				
		JE/CT	OE/CT	JE	OSE	OE
0	G	+++	+++	*	-/+	-
	H	+++	+++	*	*	-
	I	+++	++	*	*	-
	J	++++	++++	*	+	-
	K	++++	+++	++/+++	*	-
	median	+++	+++	++/+++	-/+	-
3	G	++++	++++	++/+++	-/+	-
	H	++++	++++	++++	-/+	-
	I	++	+++	++/+++	-/+	-
	J	+++	+++	*	-	-
	K	+++	+++	+++	-/+	-
	median	+++	+++	+++	-/+	-
7	G	++	+++	+++	-/+	-
	H	+++	++	*	*	-/+
	I	+++	+++	+++	-/+	-
	J	+++	+++	++	+	+
	K	++++	++++	*	-/+	-/+
	median	+++	+++	+++	-/+	-/+
10	G	+++	+++	*	*	-
	H	+++	++++	*	*	-
	I	+++	+++	*	-/+	-/+
	J	++	+++	++	-	-
	K	+++	+++	++++	-/+	-
	median	+++	+++	+++	-/+	-

* missing value

Table 3.14 VCAM-1 staining intensity for the five subjects during the 10-day experimental gingivitis study.

Day	Subject	Intensity of VCAM-1 staining	
		JE/CT	OE/CT
0	G	+	+
	H	*	*
	I	+	+
	J	+	+
	K	+	+
	median	+	+
3	G	+	+
	H	-/+	-/+
	I	+	+
	J	+	+
	K	+	+
	median	+	+
7	G	+	+
	H	+	+
	I	+	+
	J	+	+
	K	+	+
	median	+	+
10	G	+	+
	H	*	*
	I	+	+
	J	-/+	-/+
	K	+	+
	median	+	+

* missing value

within the same biopsy, ICAM-1 a 40% decrease and VCAM-1 exhibiting no difference in JE/CT when compared with OE/CT staining (day 10 compared with day 0) (Table 3.14).

ELAM-1 and ICAM-1 positive vessels were located in the same areas as previously described for the 21 day study (section 3.1.1.3), with VCAM-1 staining seen only within the connective tissue areas (JE/CT and OE/CT). VCAM-1 staining within JE/CT and OE/CT exhibited equivalent staining intensity in both areas (Fig. 3.30).

Full data sets were available for all 5 subjects (G-K) and the Friedman's test was carried out on ELAM-1 and ICAM-1 staining intensity within JE/CT and OE/CT (as VCAM-1 staining did not differ when JE/CT was compared with OE/CT). Only JE/CT staining of ELAM-1 demonstrated a significant day effect ($p=0.015$) and on further analysis (sign test, Minitab statistical package) the difference in staining intensity appeared after 3 days of plaque accumulation; for all 5 subject ELAM-1 staining within JE/CT decreased with time. No subject effect was evident for any of the adhesion molecules.

Spearman's rank correlation coefficient for association between JE/CT and OE/CT staining demonstrated that ELAM-1 staining ($r=0.799$, $p<0.0001$) correlated better than ICAM-1 staining ($r=0.562$, $p=0.01$).

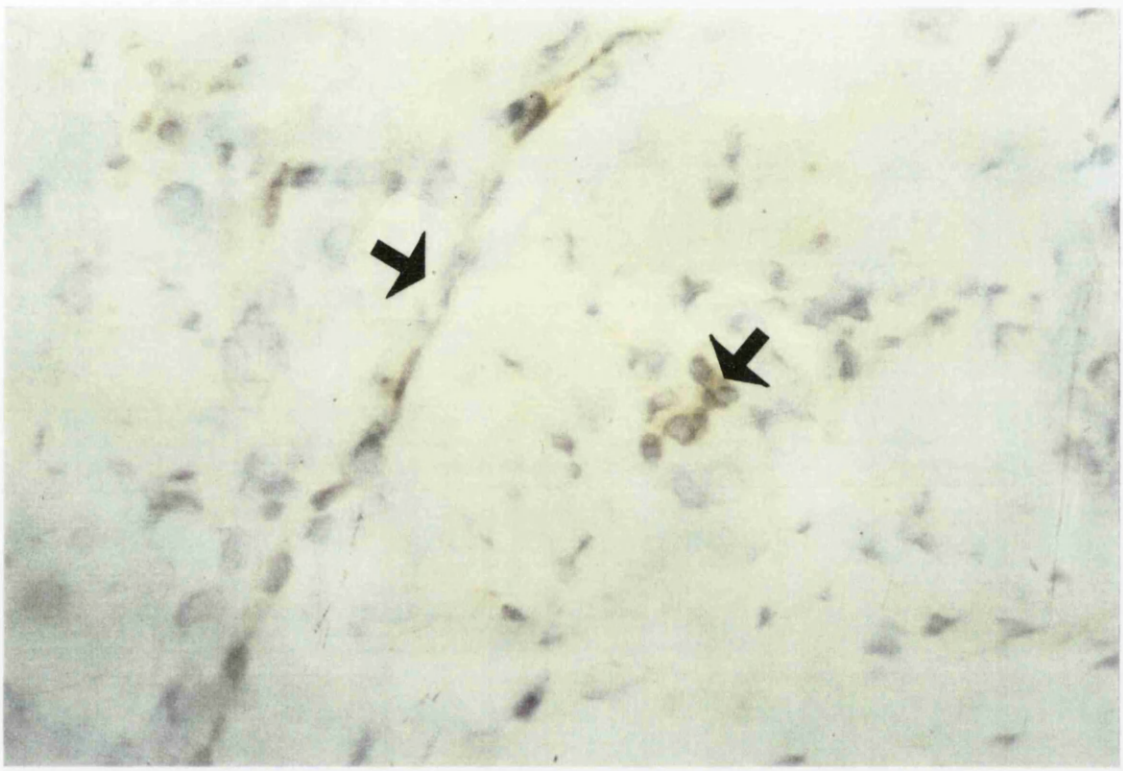

Figure 3.30 Weakly staining VCAM-1^{+} vessels within the connective tissue (solid arrows) (Immunoperoxidase, mag. x500).

The percentage of positive vessels found within the connective tissue compartment was calculated for all 3 adhesion molecules (Figs. 3.31-3.33). Statistical analysis was carried out using repeated measures analysis of variance (MANOVA, SPSS). For all 3 adhesion molecules day had no statistically significant effect, whereas subject had a significant effect ($p<0.05$) (MANOVA, SPSS) on the percentage of positive vessels.

3.1.2.5 LFA-1$^+$ leucocytes

CD11a$^+$ (LFA-1$^+$) cells were present within gingival tissue throughout the 10-days of gingival inflammation. Cells were preferentially located within the JE/CT compared with OE/CT at day 0, 3, 10 ($p<0.05$ in all cases) (Mann-Whitney, Minitab) (Fig. 3.34). The number of positive cells within JE/CT area and OE/CT area, when analysed by MANOVA (SPSS), demonstrated no significant change in numbers with days of oral hygiene abstention or subject variation.

CD11a$^+$ cells were also seen within epithelium, these IEL were located basally at day 0 (Fig. 3.35a), only 3 out of the 5 biopsies on day 3 had basally located IELs, 2 out of the 5 day 7 biopsies had IELs basally, 1 out of 5 had IELs within the basal and stratum spinosum (Fig. 3.35b) and the remaining 2 biopsies had no demonstratable IELs. By day 10 very few cells were seen within the OE. CD11a$^+$ cells were also seen located around the rete pegs with some cells also clustering around OE rete peg tips (Fig. 3.36).

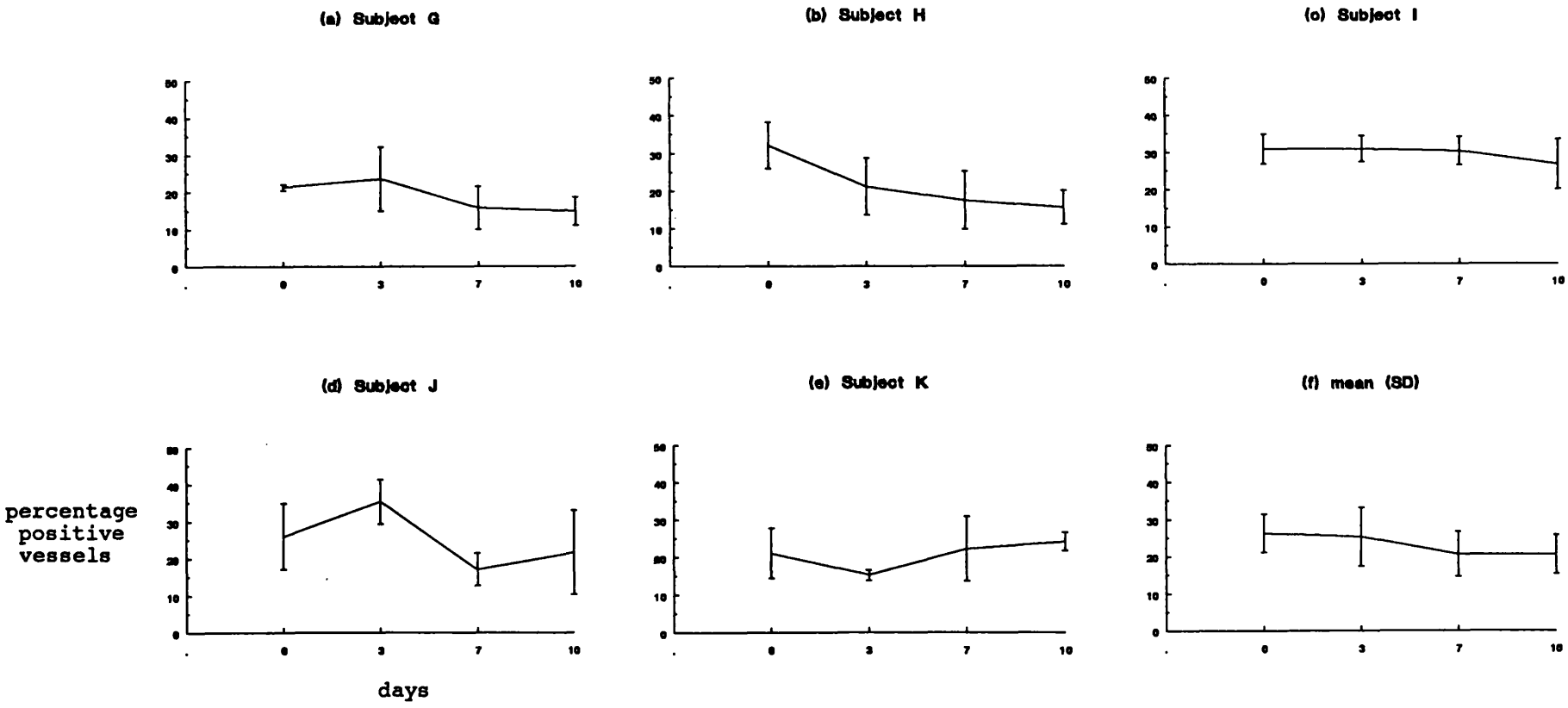

Figure 3.31 Shows the percentage of ELAM-1$^+$ positive blood vessels plotted against the development of gingival inflammation. Graphs (a)-(e) show changes in the percentage of ELAM-1$^+$ vessels for subjects G-K respectively. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

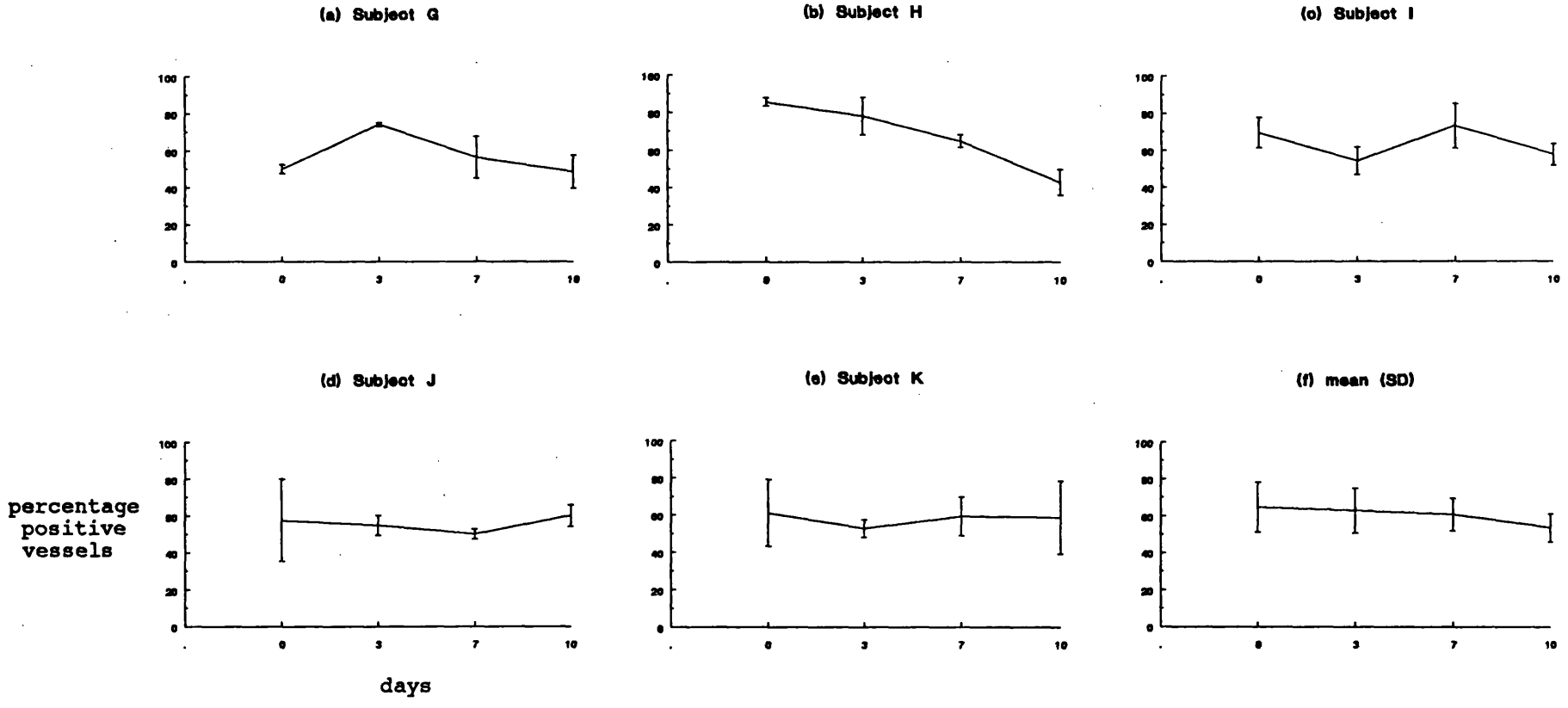

Figure 3.32 Shows the percentage of ICAM-1$^+$ positive blood vessels plotted against the development of gingival inflammation. Graphs (a)-(e) show changes in the percentage of ICAM-1$^+$ vessels for subjects G-K respectively. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

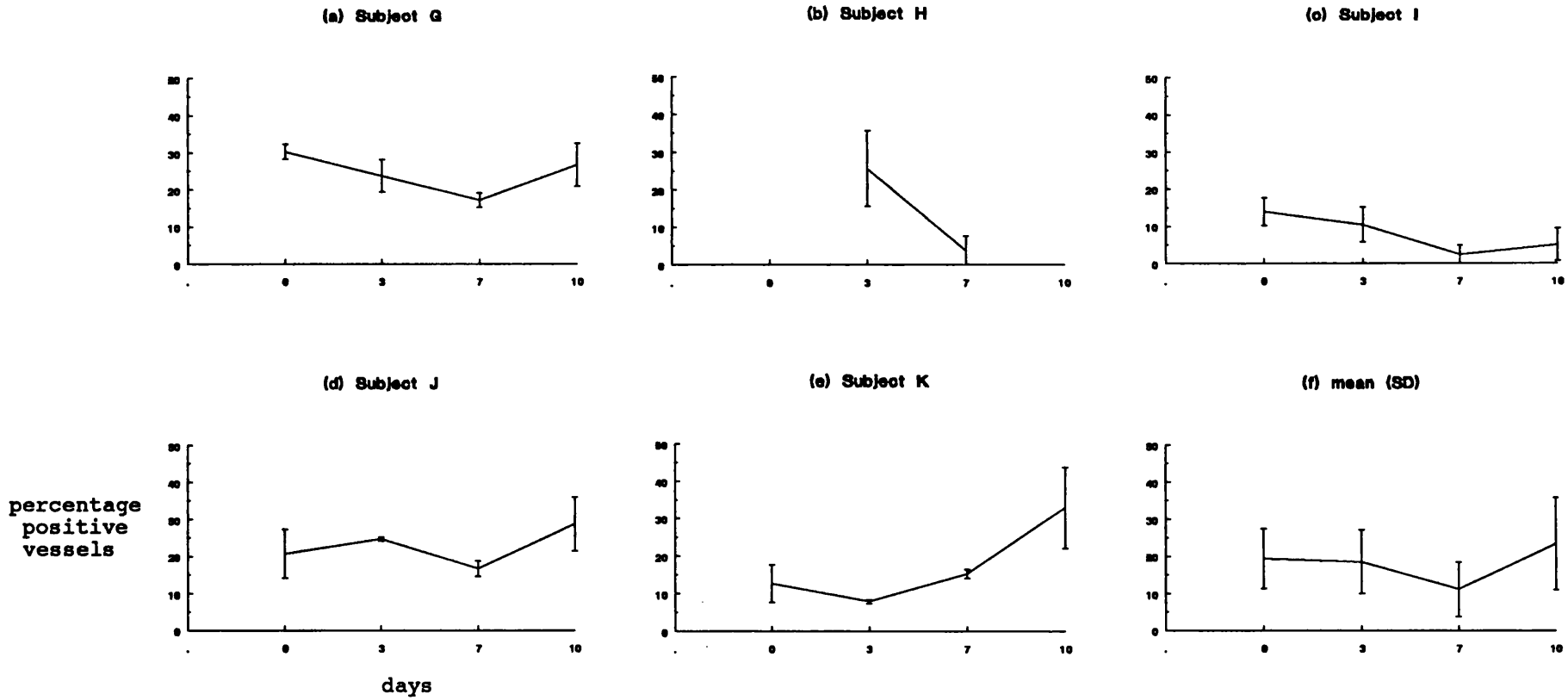

Figure 3.33 Shows the percentage of VCAM-1$^+$ positive blood vessels plotted against the development of gingival inflammation. Graphs (a)-(e) show changes in the percentage of VCAM-1$^+$ vessels for subjects G-K respectively. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

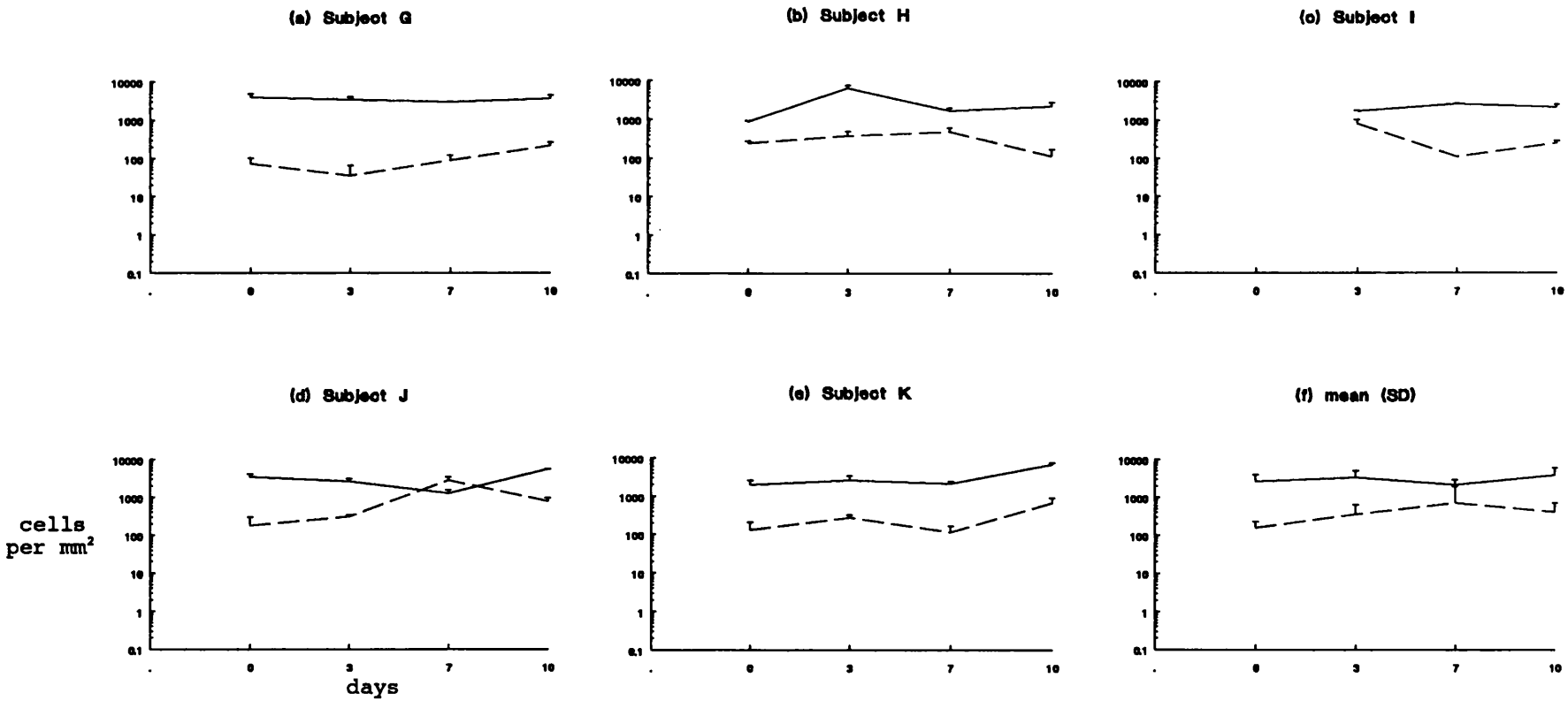

Figure 3.34 Graphs (a)-(e) show changes within LFA-1$^+$ cell (CD11a$^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects G-K respectively, during the 10-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

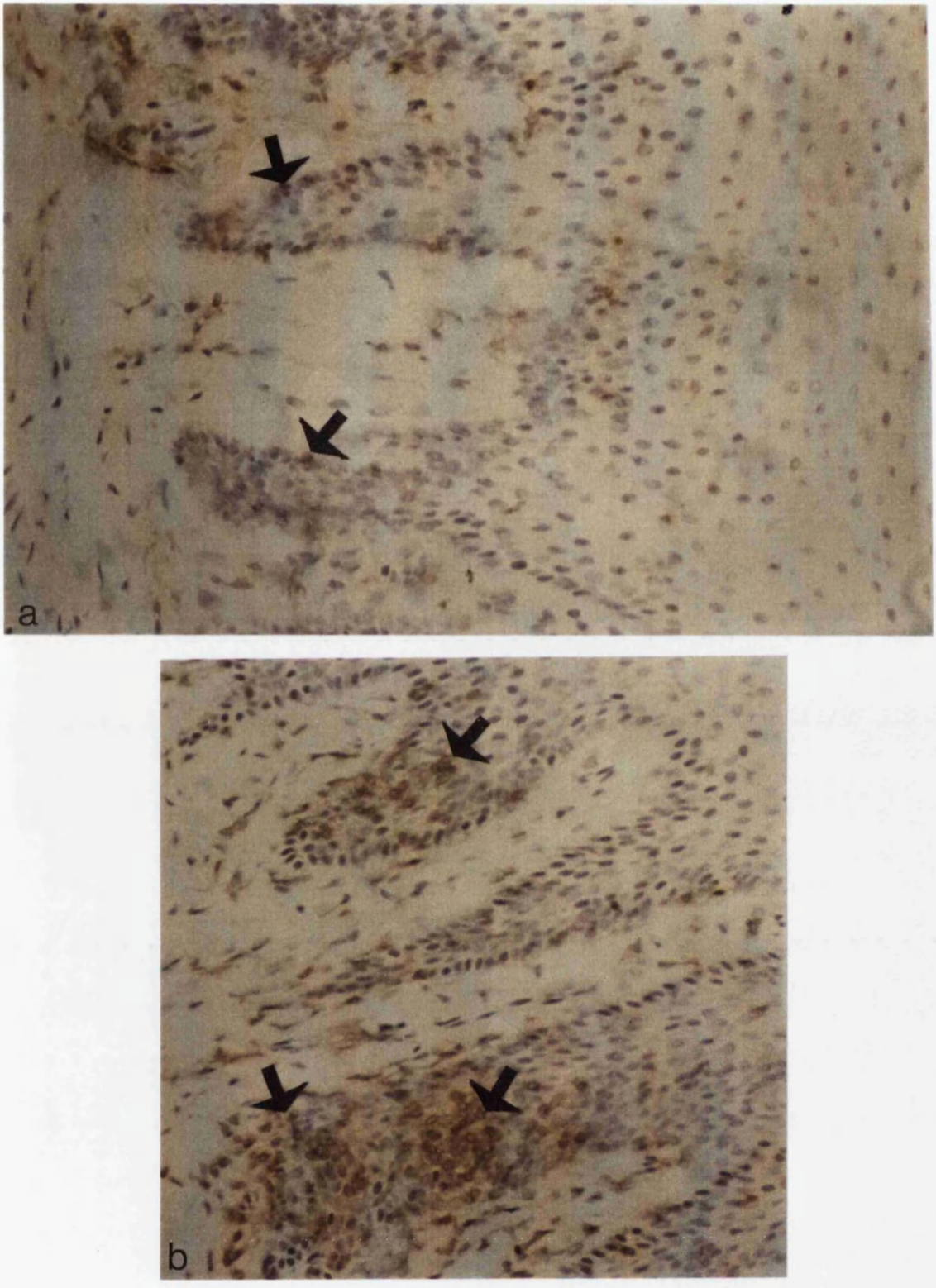

Figure 3.35 (a) Day 0 biopsy showing LFA-1^{+} (CD11a^{+}) intraepithelial cells within the basal layers of the oral epithelium (solid arrows); **(b)** Day 7 biopsy showing intraepithelial cells within the basal and stratum spinosum layers of the oral epithelium (solid arrows) (Immunoperoxidase, mag. x200).

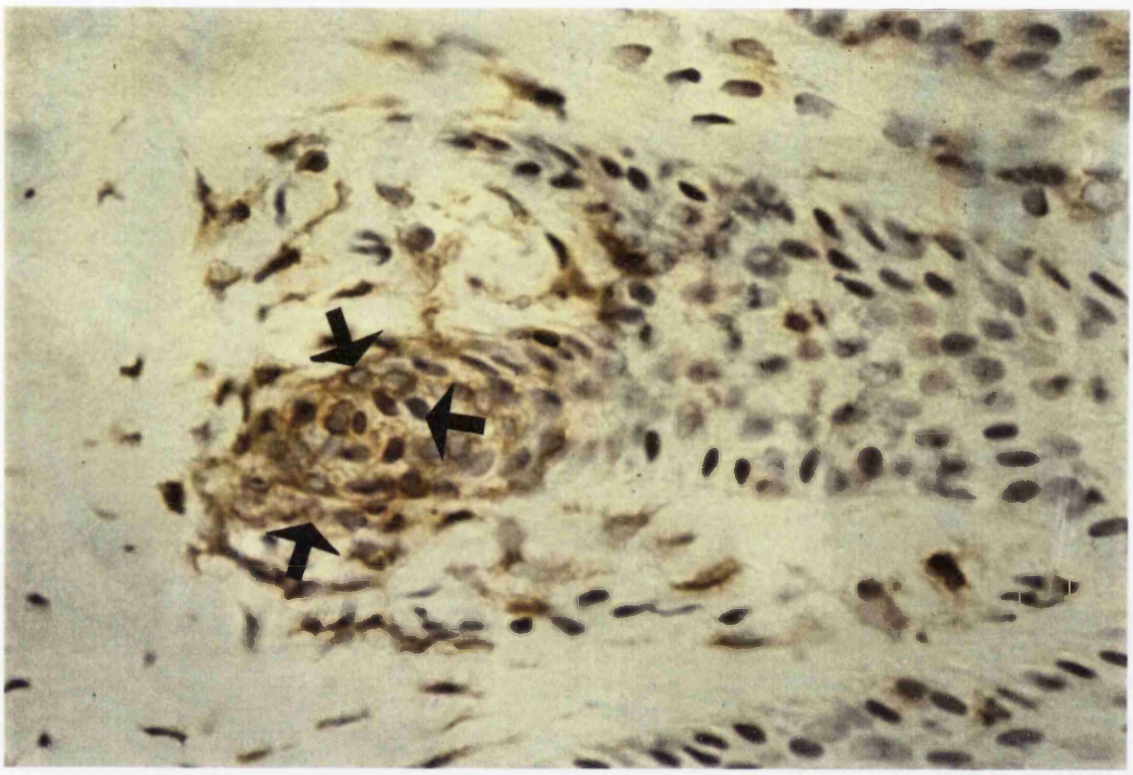

Figure 3.36 Day 3 biopsy showing LFA-1 ($CD11a^+$) cells clustered around the oral epithelium rete peg tip (solid arrows) (Immunoperoxidase, mag. x500).

When $CD11a^+$ cell numbers were converted to grading scores, no relationship was demonstrated with ICAM-1 grading within the same areas (JE/CT and OE/CT) ($r=-0.053$ and $r=-0.194$ respectively, Spearman's rank correlation coefficient, Minitab).

3.1.2.6 Neutrophils

PMNs were graded within the 4 areas of the gingival biopsies studied (JE/CT, OE/CT, JE/SE and OE) (section 2.7.6.4). PMNs appeared to be located in greater number within JE/CT than OE/CT and within JE/SE than OE (Table 3.15). Only two of the clinically 'healthy' biopsies contained detectable cells within the OE, most PMNs were present at the interface between JE/SE and tooth and within JE/CT (Fig. 3.37a). Some PMNs were also noted at the base of the biopsy (Fig. 3.37b). Friedman's test (Minitab) demonstrated no statistically significant difference in changes in cellular infiltration over days or between subjects.

Comparison of intensity of ELAM-1 staining and PMN grading demonstrated that within JE/CT their was a positive correlation ($r=0.406$; Spearman's rank correlation coefficients), although not statistically significant with time ($p=0.076$).

Table 3.15 PMN grading for each subject within each area during the 10-day experimental gingivitis study.

Day	Subject	PMN graded within whole area			
		JE/SE	SE/CT	OE/CT	OE
0	G	+	++	-	-
	H	+	+++	+	+
	I	+	++	-	-
	J	+	+++	+	+
	K	+	+++	+	-
	median	+	+++	+	-
3	G	++	+++	-	-
	H	+	+	+	-
	I	+	++	++	-
	J	+	++	+	-
	K	++	++	+	-
	median	+	++	+	-
7	G	++	+++	+	-
	H	*	++	-	-
	I	++	++	+	-
	J	+	+	+	-
	K	++	+	-	-
	median	++	++	+	-
10	G	+	+++	-	-
	H	+	+++	-	-
	I	+	+	-	-
	J	++	+++	+	-
	K	+	++	-	-
	median	+	+++	-	-

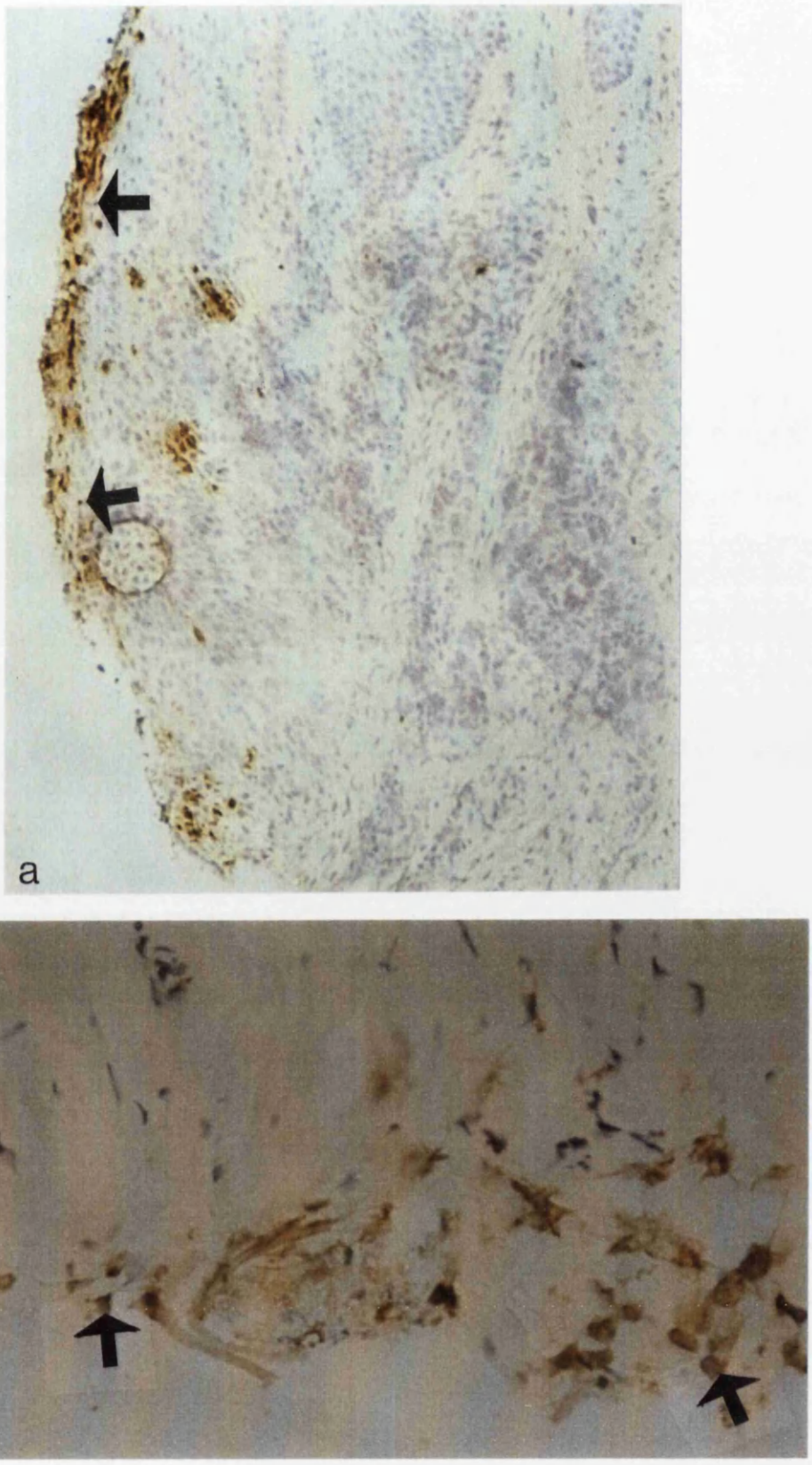

Figure 3.37 (a) PMNs located at the junctional epithelium /tooth interface of a Day 7 biopsy (solid arrows) (mag. x125); **(b)** PMNs located at the base of a Day 7 biopsy (solid arrows) (Immunoperoxidase, mag. x312.5).

3.1.2.7 T cells

$CD3^+$ T cells were located within all 4 area (JE/CT, OE/CT, JE/SE and OE). Cells within JE/CT outnumbered cells within OE/CT at day 0, 7 and 10 ($p<0.05$ in all cases) (Mann-Whitney, Minitab) (Fig. 3.38). MANOVA analysis (SPSS) showed no significant day effect on cell accumulation, although subject had effect in both areas (JE/CT and OE/CT) ($p=0.000$ and $p=0.031$ respectively).

Positive cells histologically were seen located around OE rete pegs and clustered around OE rete peg tips (seen in all biopsies). IEL at day 0 were located within the basal and stratum spinosum layers, by day 3 IEL were only located within the basal layers and by day 7 and 10 a spread of positive cells to the stratum spinosum and stratum granulosum was seen (Fig. 3.39).

$CD3^+$ IELs were present when no $CD11a^+$ IELs were seen within biopsy sections. CD11a IELs were mostly suprabasally and basally located with very few cells seen within the spinous cell layer. $CD3^+$ IELs were seen within all layers (basal, spinous and granular).

3.1.2.8 T cell subsets - T memory cells

Within clinically 'healthy' gingiva T memory cells (UCHL-1^+) were noted in abundance within the connective tissue areas (JE/CT and OE/CT) (Fig. 3.40). Significantly higher numbers of cells were found within JE/CT than OE/CT at all

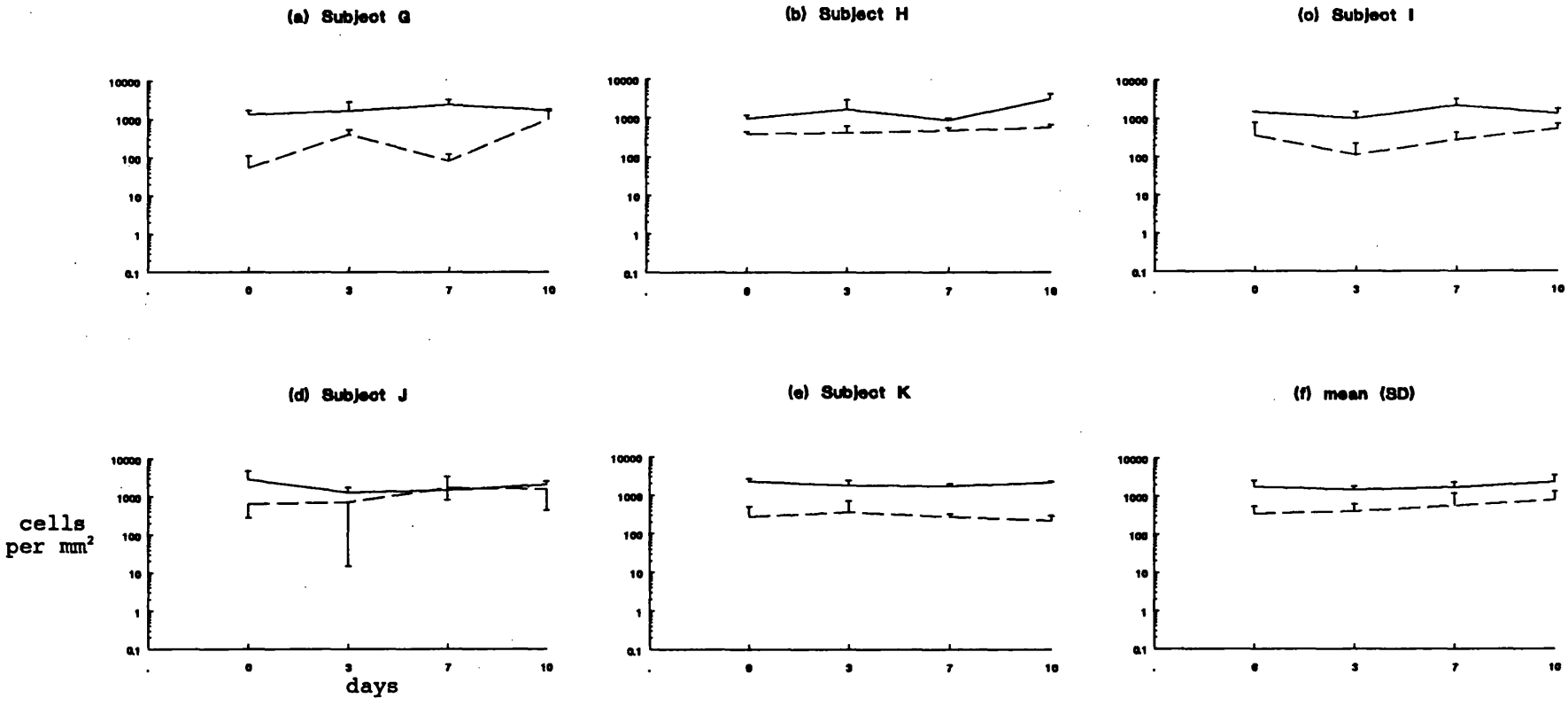

Figure 3.38 Graphs (a)-(e) show changes within T-cell ($CD3^{+}$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects G-K respectively, during the 10-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

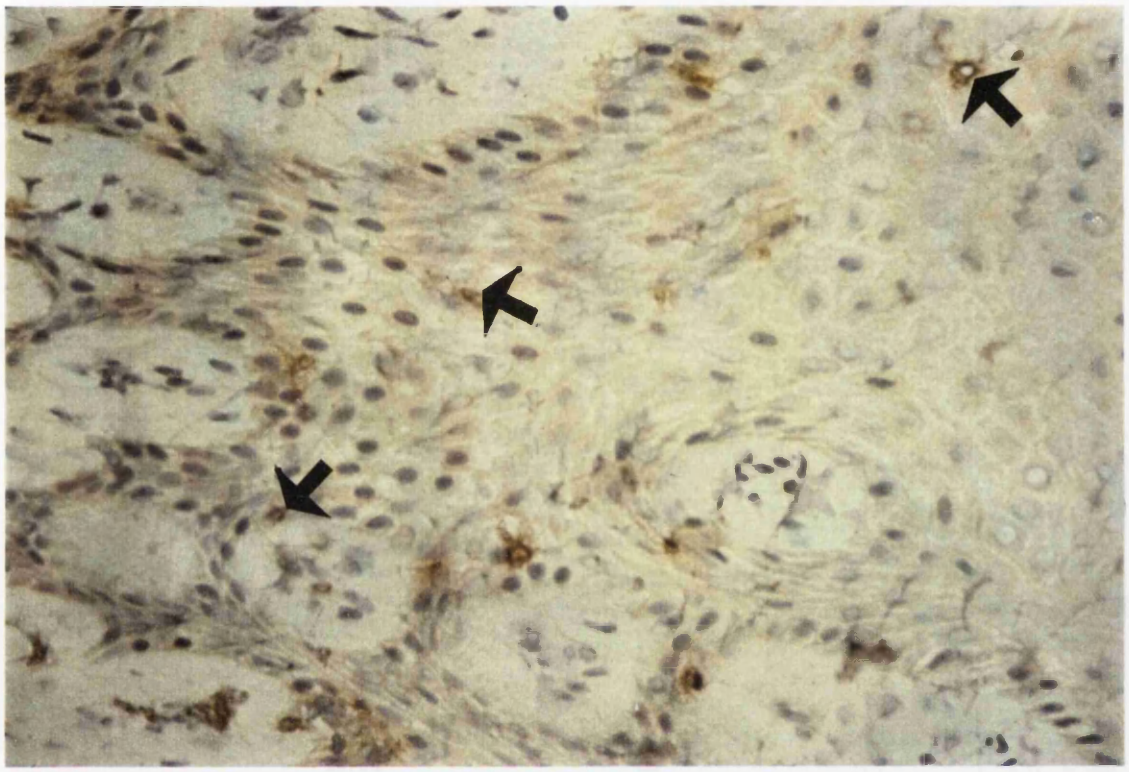

Figure 3.39 Day 7 biopsy showing $CD3^+$ T-cells with the basal, stratum spinosum and stratum granulosum layers of the oral epithelium (solid arrows) (Immunoperoxidase, mag. x312.5).

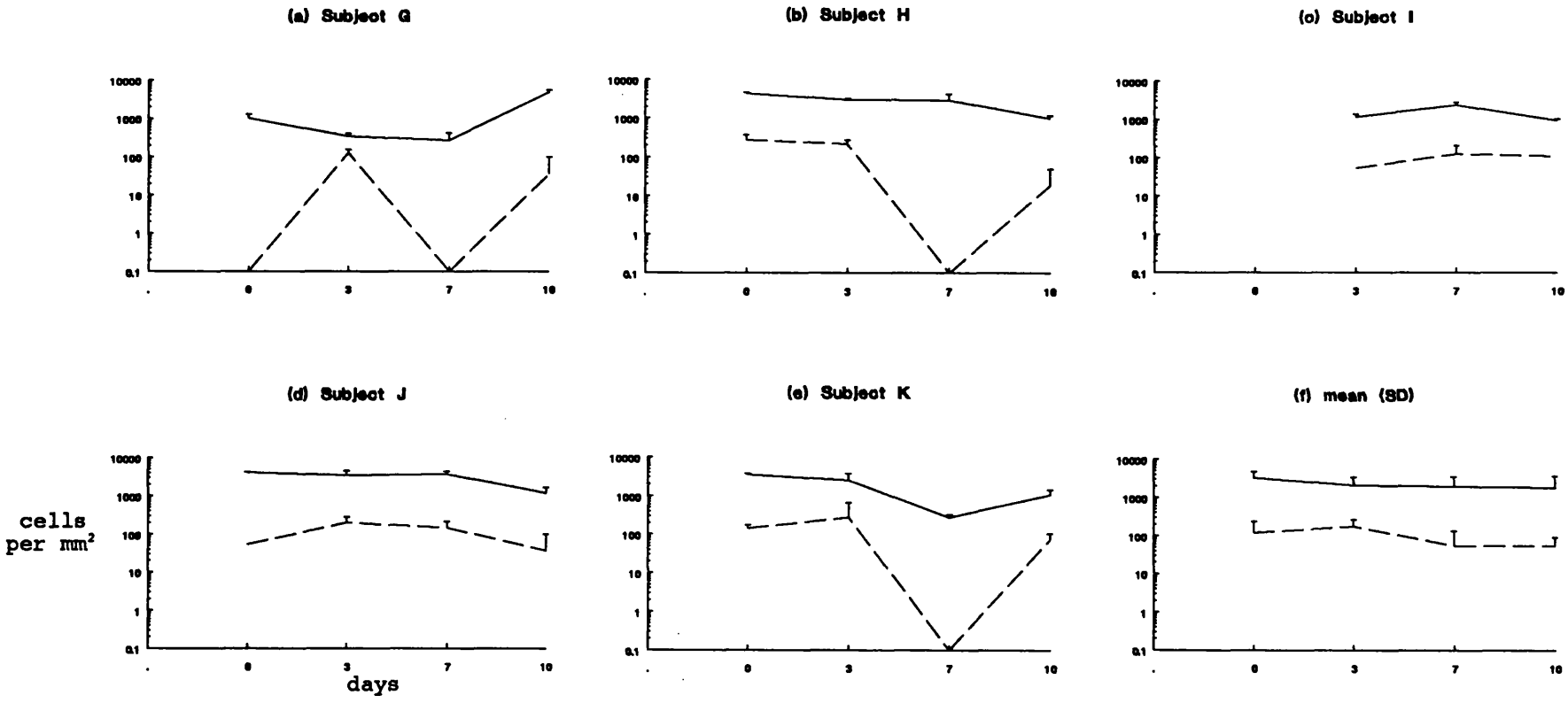

Figure 3.40 Graphs (a)-(e) show changes within memory T-cell (UCHL-1$^+$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects G-K respectively, during the 10-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

time points ($p<0.05$ in all cases) (Mann-Whitney, Minitab). T memory IEL were found within the basal layers of one biopsy only, the remaining biopsies contained no IELs.

Repeated measures analysis of variance showed that day had no effect on T memory cell accumulation within JE/CT, but did have a significant effect within OE/CT ($p=0.043$) (MANOVA, SPSS). On further analysis a decrease in T memory cell numbers was noted between day 3 and day 7 ($p=0.04$) and between day 3 and day 10 ($p=0.008$). Subject had a significant affect within both JE/CT and OE/CT ($p=0.016$ and $p=0.008$). Since multiple comparisons were used a Bonferroni correction of the significance level has to be employed which means the standard 5% level of significance is reduced to a $p<0.01$ threshold for declaring differences are statistically significant (section 2.7.7.1).

3.1.2.9 T cell subsets - T naive/virgin cells

Positive cells ($2H4^+$) were seen throughout the 10-days of oral hygiene abstention. Cells were located within JE/CT, OE/CT and JE/SE, no cells were seen within OE i.e. no IELs. Cell numbers in JE/CT, when compared with OE/CT (Fig. 3.41), demonstrated statistically significantly elevated numbers only at day 3 ($p<0.05$, Mann-Whitney, Minitab), though there is a trend for JE/CT naive cell numbers to be elevated over OE/CT numbers in all biopsies.

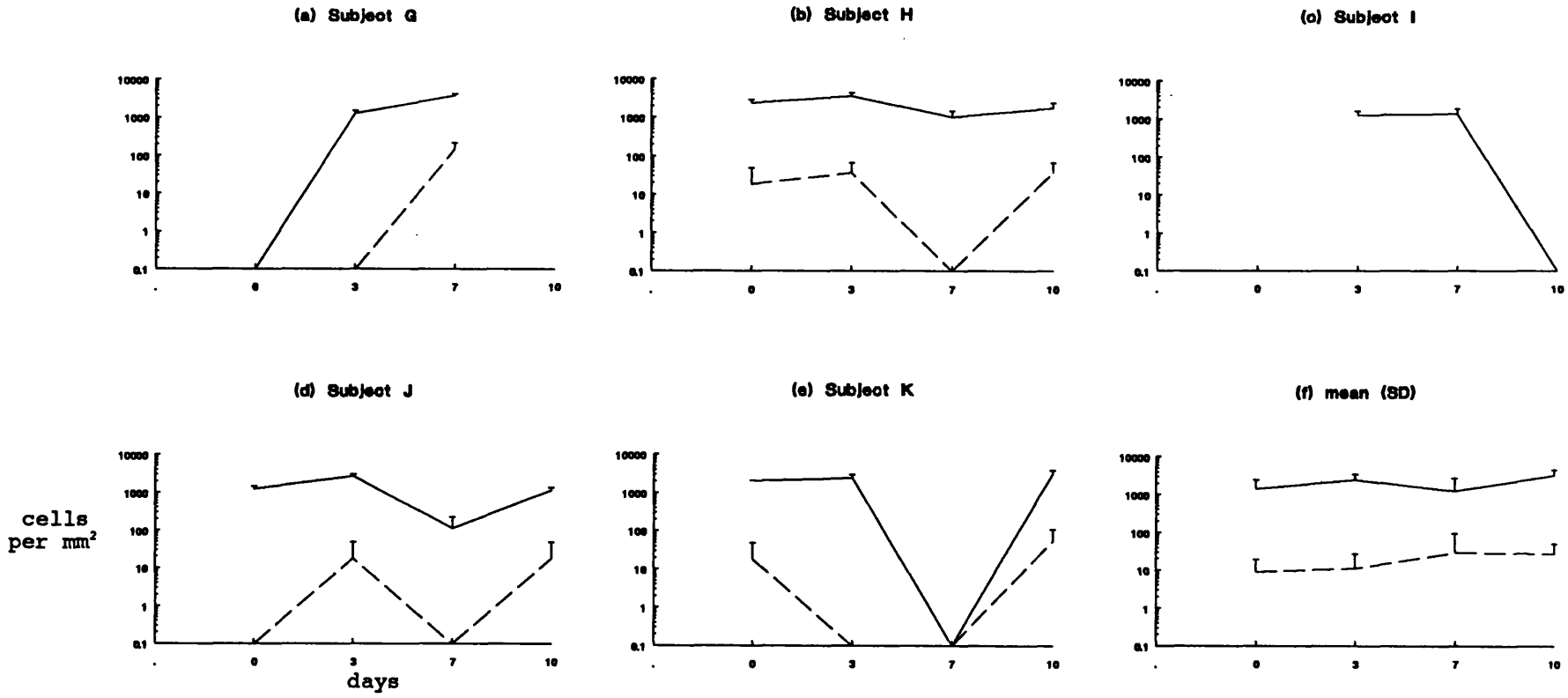

Figure 3.41 Graphs (a)-(e) show changes within naive/virgin T-cell ($2H4^{+}$) numbers within the connective tissue subjacent to the junctional epithelium (JE/CT) (——) and subjacent to the oral epithelium (OE/CT) (---) for subjects G-K respectively, during the 10-day experimental gingivitis study. Each point represents the mean and standard deviation of 3 readings. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

MANOVA analysis demonstrated that day has a significant affect on naive cell accumulation within the JE/CT ($p=0.019$) and on further analysis it was shown that a decrease in cell number was seen between day 0 and day 7 ($p=0.041$) and between day 3 and day 7 ($p=0.000$) (3 full data sets, subjects H, J and K). Since multiple comparisons were used a Bonferroni correction has to be employed, $p<0.01$ (section 2.7.7.1). Subject had a significant effect within JE/CT ($p=0.008$) and within OE/CT ($p=0.016$).

Comparisons of T naive/virgin and T memory cells (Tn/v:Tmem ratio) within JE/CT and OE/CT over the four time points were reasonably consistent over time except T memory cells were significantly elevated over T naive/virgin at day 3 within the OE/CT area ($p=0.0112$) (Mann-Whitney, Minitab), i.e. when very few T naive/virgin cells were present.

3.1.2.10 Langerhans cells

$CD1a^+$ LCs were seen within OE and SE of clinically 'healthy' and experimentally inflamed tissue (Fig. 3.42a). Within clinically 'healthy' tissue LCs were dendritic in appearance and located within basal, stratum spinosum and stratum granulosum layers of the OE (Fig. 3.42a). As inflammation developed a slight shift in cells towards the stratum granulosum layer was seen and by day 7 cells were still seen within basal and stratum spinosum layers but were more concentrated within the stratum granulosum and

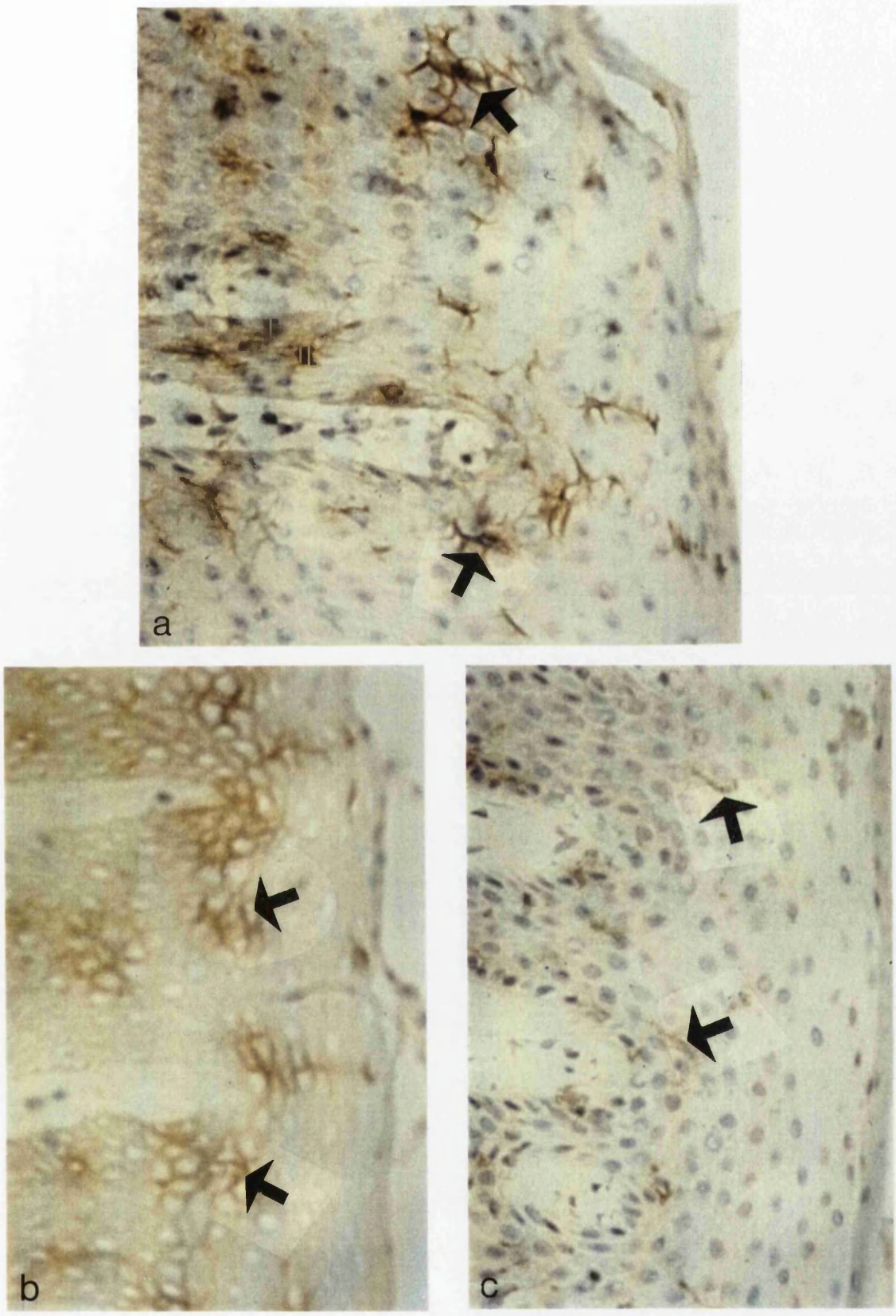

Figure 3.42 (a) Day 0 biopsy: Highly dendritic Langerhans cells within the oral epithelium (OE) (solid arrows); **(b)** Day 7 biopsy: Dendritic Langerhans cells with OE (solid arrows) and **(c)** Less dendritic Langerhans cells (solid arrows) (Immunoperoxidase, mag. x312.5).

more dendritic in appearance compared with CD1a$^+$ LCs in day 0 and day 3 tissue (Fig. 3.42b). By day 10 there was a large shift in cell location from the stratum granulosum to basal and stratum spinosum layers and LCs appeared less dendritic (Fig. 3.42c). In a few biopsies when JE was present CD1a$^+$ cells were noted (Fig. 3.43).

HLA-DR identified cells with the same dendritic morphology as CD1a$^+$ cells and were identified in serial sections.

Day had no effect on CD1a$^+$ or HLA-DR$^+$ LCs within the OE. Subject had a significant effect on CD1a$^+$ or HLA-DR$^+$ LCs ($p<0.001$ and $p=0.005$ respectively) (MANOVA, SPSS) (Fig. 3.44).

3.2 Organ culture

3.2.1 ELAM-1 - percentage of positive vessels

The percentage of ELAM-1$^+$ vessels are presented as previously discussed in (section 2.7.6.7). Briefly, control results are expressed as percentage of positive vessels and cytokine-enriched media results as percentage net change after correction for background.

3.2.1.1 No addition

Changes in the percentage of ELAM-1 positive vessels with time can be seen in Figure 3.45a. The percentage of ELAM-1$^+$ vessels changed very little with incubation time, although a slight peak occurred at 48 hours.

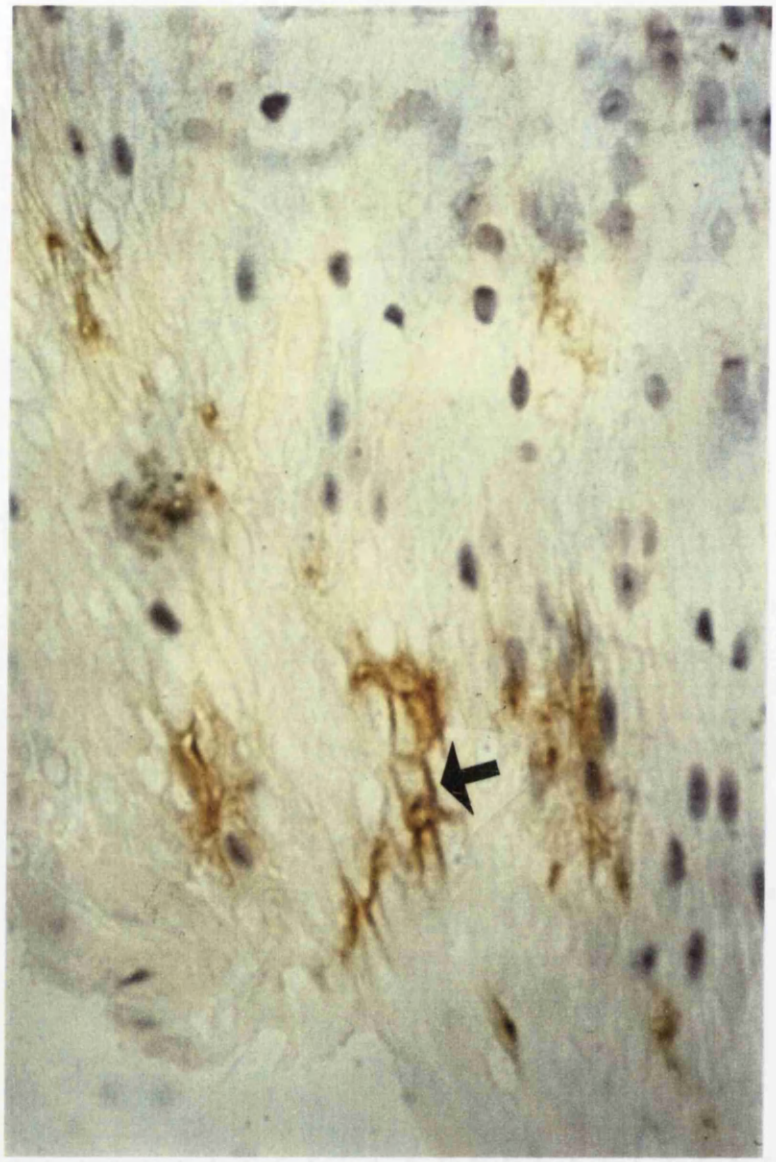

Figure 3.43 A day 10 biopsy with a CD1a^{+} Langerhans cell within the junctional epithelium (solid arrow) (Immunoperoxidase, mag. x500).

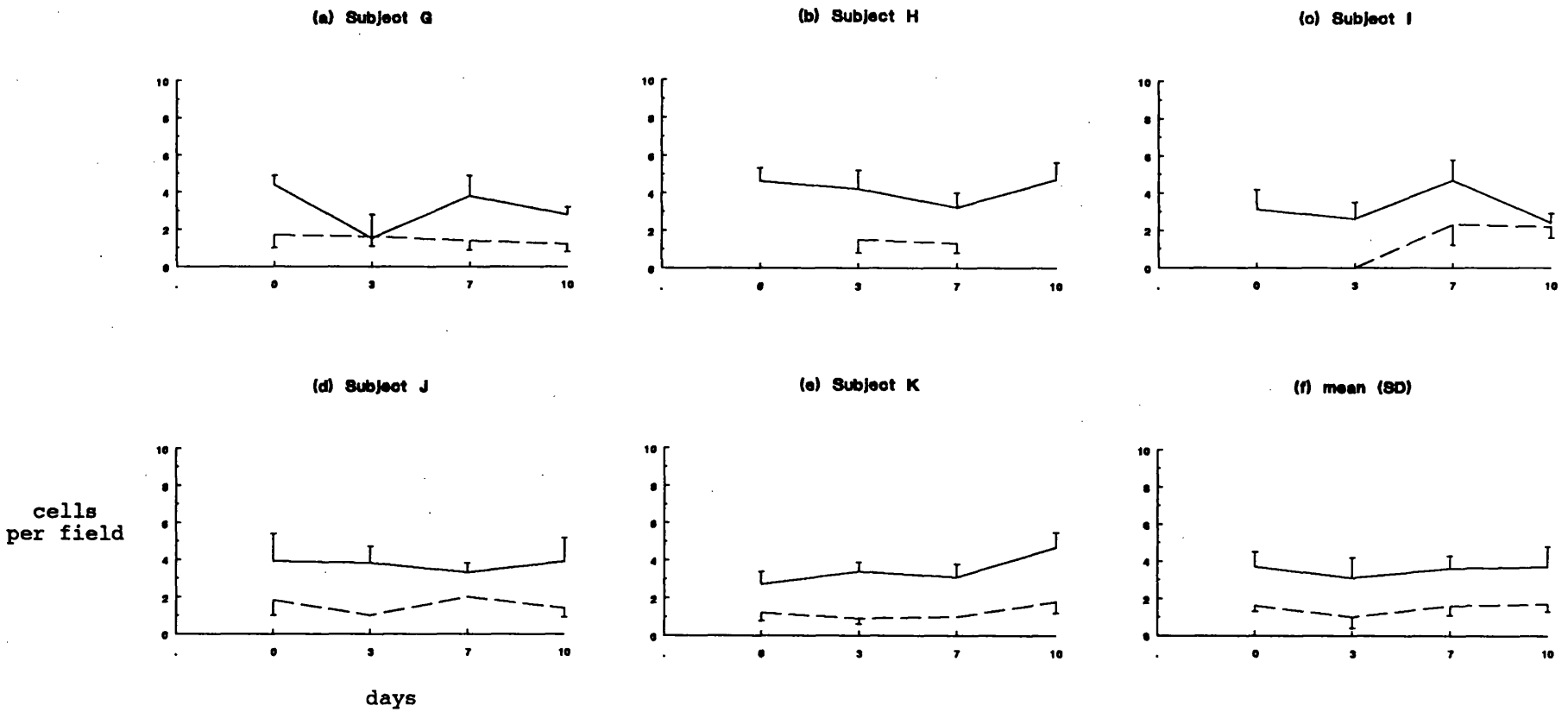

Figure 3.44 Graphs (a)-(e) show changes within Langerhans cells numbers, CD1a$^+$ (——) and HLA-DR$^+$ (---), within the oral epithelium for subjects G-K respectively, during the 10-day experimental gingivitis study. Each point represents the mean and standard deviation of 10 fields. Graph (f) depicts the mean and standard deviation of the individual subjects means (n=5). The x-axis and y-axis legends for all the graphs are identical.

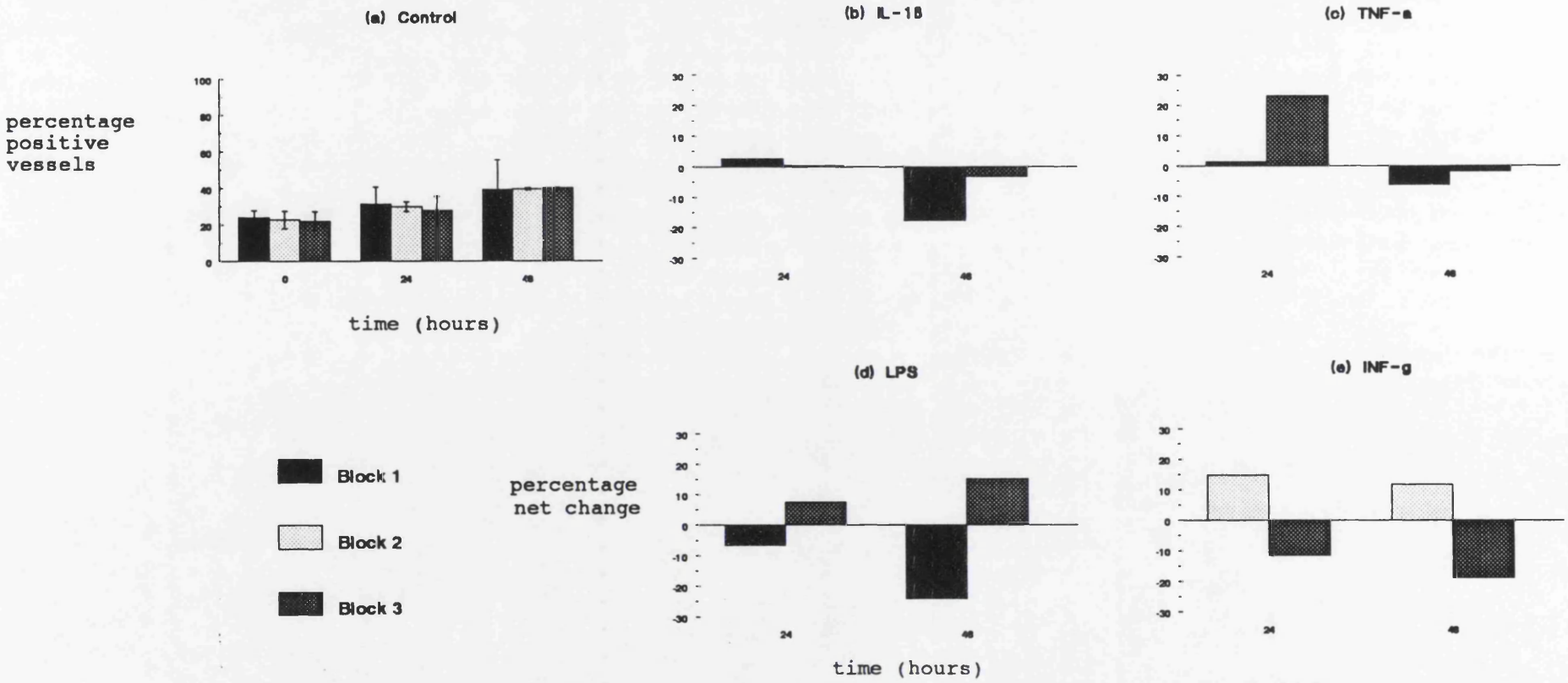

Figure 3.45 Graph (a) shows changes in ELAM-1 percentage positive vessels with incubation time. Each point represents the mean and standard deviation of 3 readings. Graphs (b)-(e) show the calculated percentage net change in the number of ELAM-1$^+$ vessels in various cytokines. The cytokines used were (b) interleukin-1β (IL-1β); (c) tumour necrosis factor-α (TNFα); (d) lipopolysaccharide (LPS) and (e) interferon-γ (INFγ).

3.2.1.2 Interleukin-1ß

IL-1ß at 24 hours increased the percentage of positive vessels and at 48 hours percentage positivity had decreased in both blocks 1 and 3 (Fig. 3.45b).

3.2.1.3 Tumour necrosis factor-α

TNFα increased the percentage of ELAM-1$^+$ vessels seen after 24 hours incubation but by 48 hours the percentage had decreased within both blocks 1 and 3 (Fig. 3.45c)

3.2.1.4 Lipopolysaccharide

LPS had different effects in both blocks, at 24 and 48 hours, percentages of ELAM-1$^+$ vessels increased in one block 3 and decreased in block 1 (Fig. 3.45d).

3.2.1.5 Interferon-γ

IFN-γ displayed similar effects as LPS. Block 2 displayed increased ELAM-1$^+$ positivity at 24 and 48 hours and block 3 decreased (Fig. 3.45e) which was opposite to the effects seen due to LPS (Fig. 3.45d).

3.2.2 ELAM-1 staining intensity

Staining intensity results were processed and presented as previously discussed (section 2.7.6.7). Briefly, control results are expressed as intensity changes over time and cytokine-enriched media results as net change in staining intensity after correction for background.

3.2.2.1 No addition

Changes in ELAM-1 staining intensity within blocks differed. Block 1 exhibited no change in staining intensity with time, whereas block 2 staining intensity remained stable until 24 hours and then displayed increased staining intensity by 48 hours and then finally block 3 displayed minimal staining initially (24 hours) followed by a large increase by 48 hours (Fig. 3.46a).

3.2.2.2 Interleukin-1ß

IL-1ß caused ELAM-1 staining intensity within block 3 to increase after 24 hours and then decrease after 48 hours whereas block 1 displayed no change in ELAM-1 staining intensity after 24 hours and decreased intensity after 48 hours (Fig. 3.46b).

3.2.2.3 Tumour necrosis factor-α

TNFα displayed similar effects on blocks 1 and 3 at 24 hours as IL-1ß, with one block displaying increased ELAM-1 staining intensity and the other block exhibiting no change in intensity. After 48 hours incubation both blocks displayed no change in staining intensity when compared with the controls (Fig. 3.46c).

3.2.2.4 Lipopolysaccharide

After 24 hours incubation with LPS both blocks (1 and 3) demonstrated increased ELAM-1 staining intensity but after 48 hours block 3 continued to exhibit increased staining

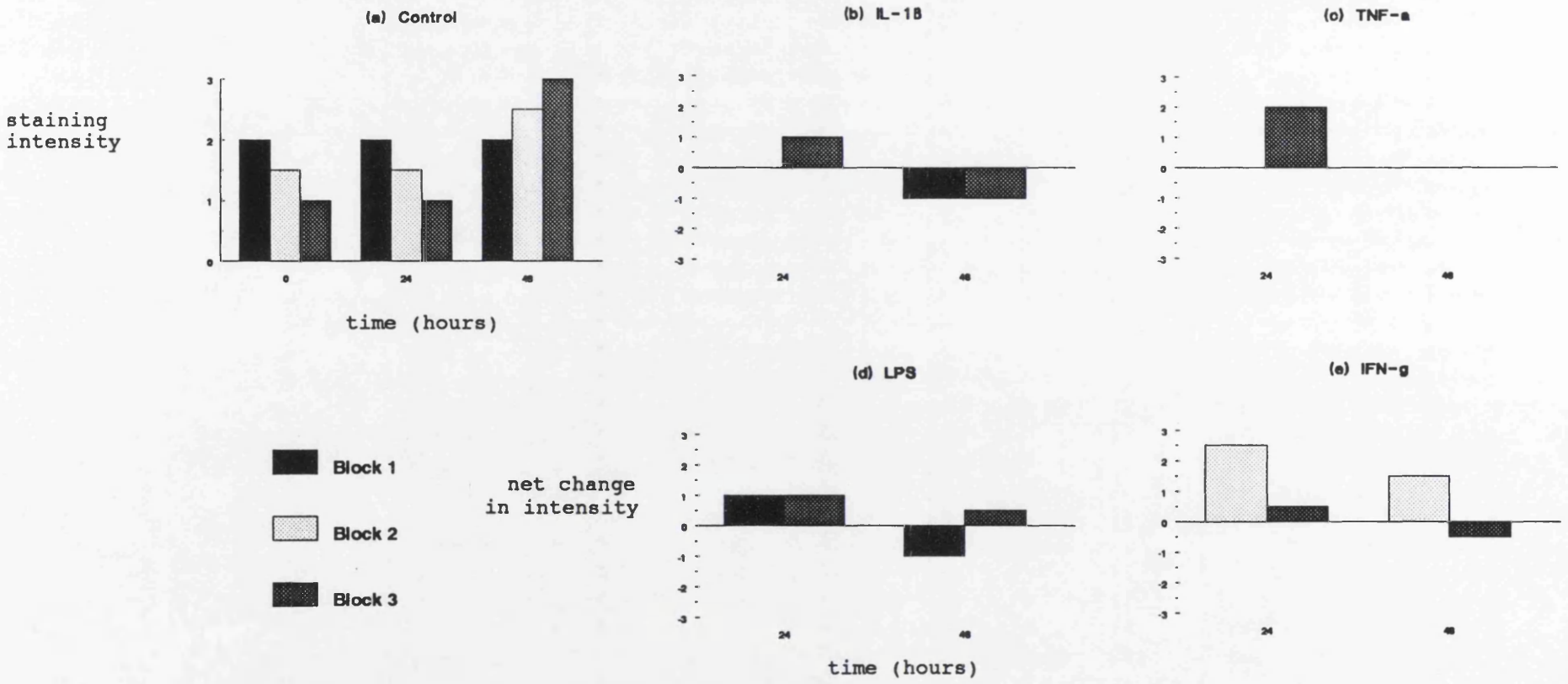

Figure 3.46 Graph (a) shows changes in ELAM-1 staining intensity with incubation time. Each point represents the mean of 3 gradings. Graphs (b)-(e) show the calculated net change in the intensity of ELAM-1 staining using various cytokines. The cytokines used were (b) interleukin-1β (IL-1β); (c) tumour necrosis factor-α (TNFα); (d) lipopolysaccharide (LPS) and (e) interferon-γ (INFγ).

intensity and block 1 decreased in ELAM-1 staining intensity when compared with the controls (Fig. 3.46d).

3.2.2.5 Interferon-γ

IFN-γ at 24 hours exhibited similar effects on ELAM-1 staining intensity after 24 hours and 48 hours within both blocks (Fig. 3.46e). But the block that displayed increased staining intensity after 48 hours with LPS demonstrated decreased staining intensity with IFN-γ (block 3).

3.2.3 ICAM-1 - percentage of positive vessels

Percentage of ICAM-1 positive vessels from the organ culture studies are presented using the method discussed previously (section 2.7.6.7).

3.2.3.1 No addition

The changes in the percentage of ICAM-1$^+$ vessels with incubation time can be seen in Figure 3.47a. Over time, vessel staining remained fairly constant, with a slight increase by 48 hours.

3.2.3.2 Interleukin-1ß

Both blocks exhibited increased percentage of ICAM-1$^+$ vessels after 24 hours but after 48 hours block 1 continued to increase with block 3 decreasing in its percentage of ICAM-1$^+$ vessels (Fig. 3.47b).

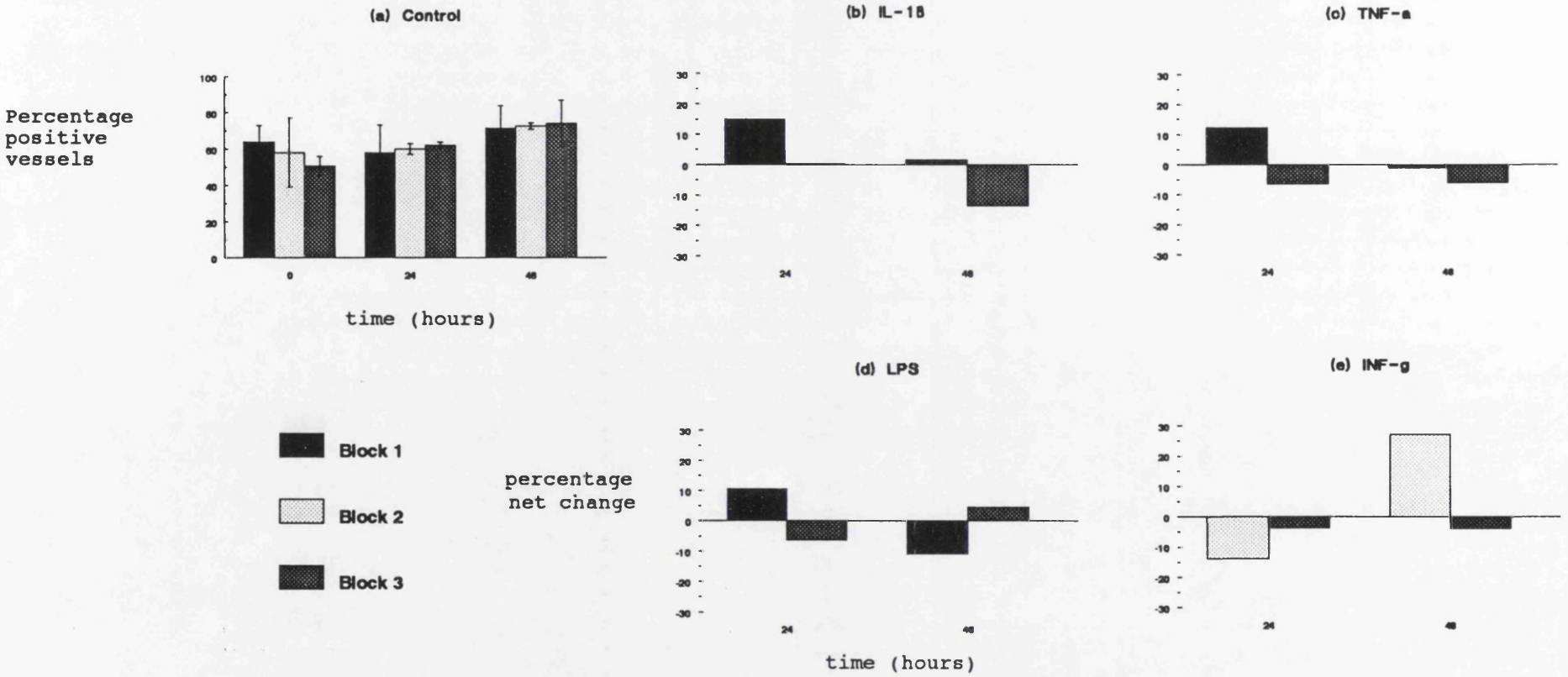

Figure 3.47 Graph (a) shows changes in ICAM-1 percentage positive vessels with incubation time. Each point represents the mean and standard deviation of 3 readings. Graphs (b)-(e) show the calculated percentage net change in the number of ICAM-1$^+$ vessels in various cytokines. The cytokines used were (b) interleukin-1β (IL-1β); (c) tumour necrosis factor-α (TNFα); (d) lipopolysaccharide (LPS) and (e) interferon-γ (INFγ).

3.2.3.3 Tumour necrosis factor-α

After 24 hours in culture, block 1 increased and block 3 decreased in the percentage of ICAM-1$^+$ vessels seen. By 48 hours both blocks had decreased percentages when compared with controls (Fig. 3.47c).

3.2.3.4 Lipopolysaccharide

LPS caused opposite effects in the two blocks, after 24 hours block 1 increased whereas block 3 decreased and by 48 hours the opposite occurred. With the block that previously exhibited an increase now decreasing and vice-versa (Fig. 3.47d).

3.2.3.5 Interferon-γ

Decreases in the percentage of ICAM-1$^+$ positive vessels at 24 and 48 hours was seen within block 3 whereas block 2 demonstrated a decrease at 24 hours and an increase at 48 hours (Fig. 3.47e).

3.2.4 ICAM-1 staining intensity

Results expressed as discussed previously (section 2.7.6.7).

3.2.4.1 No addition

Blocks 1 and 3 displayed moderate ICAM-1 staining intensity with block 2 displaying strong staining at 0 hours. By 24 and 48 hours all blocks displayed strong ICAM-1 staining (Fig. 3.48a).

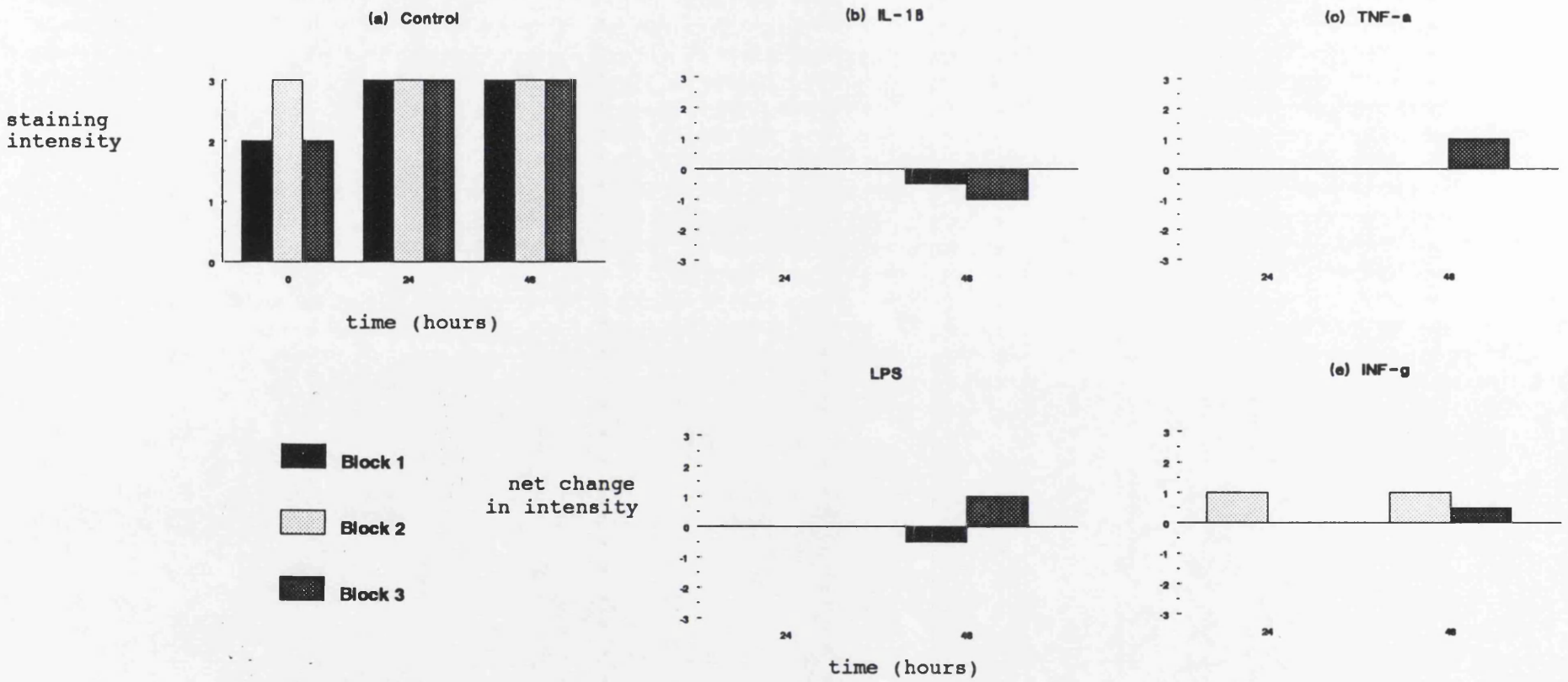

Figure 3.48 Graph (a) shows changes in ICAM-1 staining intensity with incubation time. Each point represents the mean of 3 gradings. Graphs (b)-(e) show the calculated net change in the intensity of ICAM-1 staining using various cytokines. The cytokines used were (b) interleukin-1β (IL-1β); (c) tumour necrosis factor-α (TNFα); (d) lipopolysaccharide (LPS) and (e) interferon-γ (INFγ).

3.2.4.2 Interleukin-1ß

At 24 hours, ICAM-1 staining intensity did not change within the blocks when compared with the controls, but by 48 hours both (blocks 1 and 3) exhibited decreased staining intensity (Fig. 3.48b).

3.2.4.3 Tumour necrosis factor-α

No change in staining intensity was seen after 24 hours in culture, by 48 hours block 1 still exhibited no change with block 3 increasing in ICAM-1 staining intensity (Fig. 3.48c).

3.2.4.4 Lipopolysaccharide

LPS induced no change in intensity after 24 hours incubation (Fig. 3.48d), similar changes seen with TNFα (Fig. 3.48c) and IL-1ß (Fig. 3.48b). After 48 hours, block 3 exhibited increased ICAM-1 staining intensity and block 1 decreased in staining intensity (Fig. 3.48d).

3.2.4.5 Interferon-γ

IFN-γ in block 3 demonstrated no change in ICAM-1 staining intensity and increased staining intensity in block 2 after 24 hours. But by 48 hours both blocks exhibited increased ICAM-1 staining intensity (Fig. 3.48e). Interestingly IFN-γ was the only cytokine which caused OE KC to express ICAM-1, which also demonstrated a gradient of staining from the connective tissue towards the outer aspect, as seen in Figure 3.49.

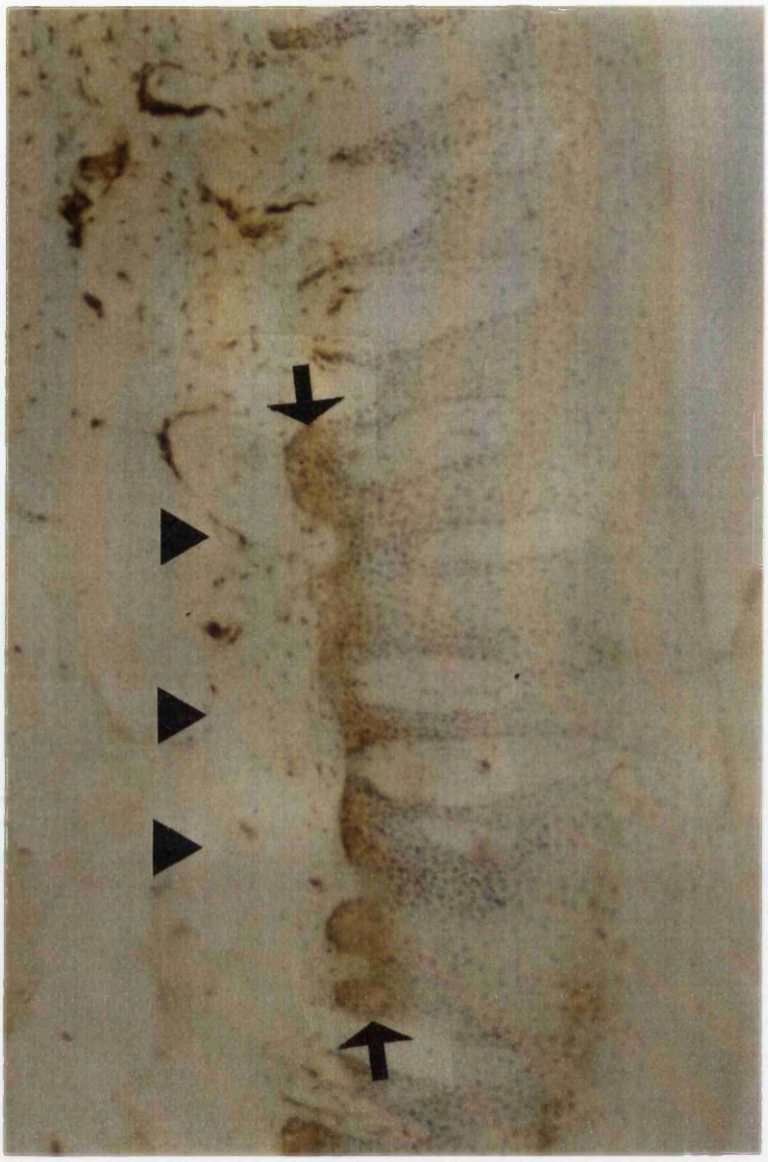

Figure 3.49 ICAM-1 staining gradient effect seen at the base of the oral epithelium rete pegs (solid arrows) caused by interferon-γ (direction of cytokine source indicated by solid triangles) (Immunoperoxidase, mag. 125).

3.3 Adhesion molecule expression in diseased tissue

Adhesion molecules, ELAM-1 and ICAM-1 were evaluated in diseased tissue, the percentage of positive vessels as well as the intensity of staining was appraised.

3.3.1 Adult Periodontitis

3.3.1.1 ELAM-1 expression

ELAM-1 expression in the adult periodontitis tissue was concentrated within the blood vessels. Positive staining blood vessels were preferentially located subjacent to the epithelium (Fig. 3.50) and within the cellular infiltrate when present. In two cases ELAM-1 staining of keratinocytes was also seen. Staining intensity of the vessels varied (Table 3.16) with vessels within each section exhibiting the same intensity of staining.

The percentage of positive vessels per biopsy were also calculated and were shown to range from 18.7 to 44.1% (Table 3.17).

3.3.1.2 ICAM-1 expression

ICAM-1 demonstrated staining of vessels within areas of high cellular infiltration (Fig. 3.51). Epithelium also exhibited keratinocyte staining within the outer 2-3 cell layers, when deeper staining was seen ie 6-7 cell layers thick, an ICAM-1 gradient was noted (Fig. 3.52). One biopsy had no demonstratable ICAM-1 epithelium staining. Variable quantities of JE were available on each biopsy due

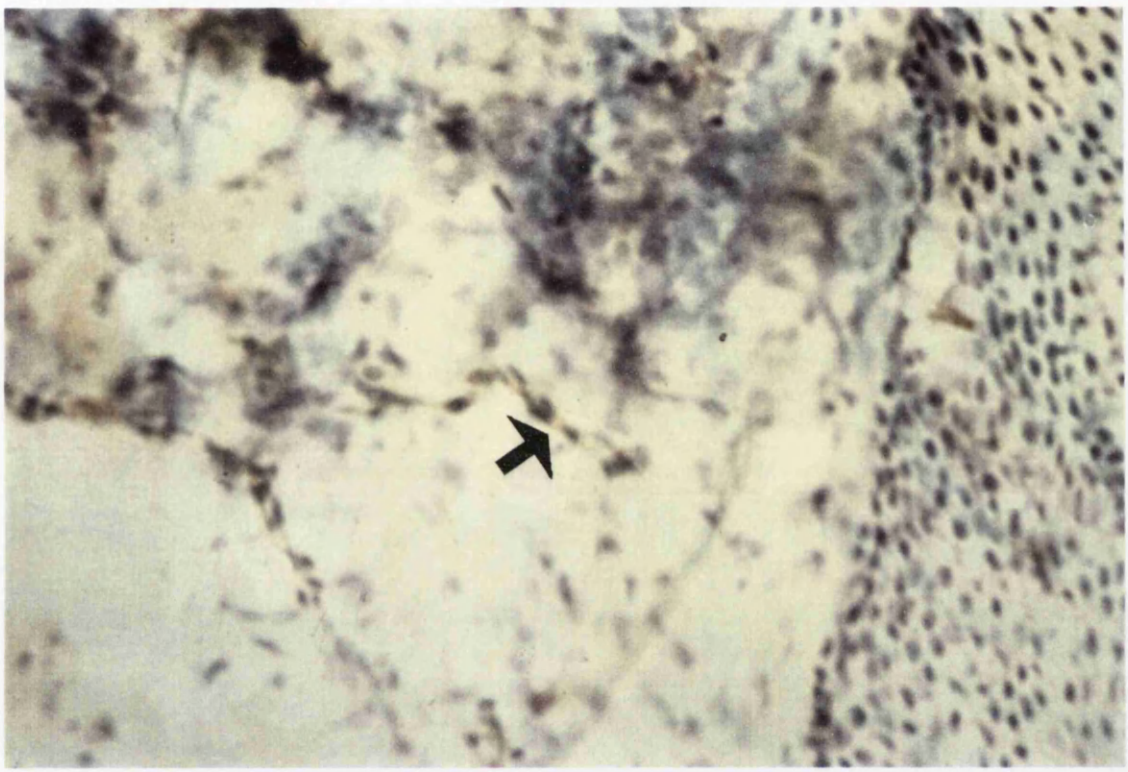

Figure 3.50 Adult periodontitis tissue: ELAM-1^{+} vessel seen subjacent to the epithelium (solid arrow) (Immunoperoxidase, mag. x312.5).

Table 3.16 ELAM-1 and ICAM-1 staining intensity in diseased tissue obtained from subjects suffering from adult periodontitis (AP), rapidly progressive periodontitis (RPP) and juvenile periodontitis (JP).

Disease	Biopsy	ELAM-1 intensity	ICAM-1 intensity
AP	1	+	*
AP	2	+	+
AP	3	++	+++
AP	4	++	++
AP	5	++	++
AP	6	+	++
AP	7	+	++/+++
AP	8	+	+++/++++
AP	9	+	+
AP	10	+/+++	++/+++
RPP	11	+	+
RPP	12	+/+++	++
RPP	13	++/+++	+++
RPP	14	+	+/++
RPP	15	++/+++	+++/++++
RPP	16	+	+++
RPP	17	+	++/+++
JP	18	+	++
JP	19	+/++	+++
JP	20	+/++	++/+++

* missing value

Table 3.17 Percentage of ELAM-1 and ICAM-1 positive blood vessels in diseased tissue obtained from subjects suffering from adult periodontitis (AP), rapidly progressive periodontitis (RPP) and juvenile periodontitis (JP).

Disease	Biopsy	% ELAM-1$^+$	%ICAM-1$^+$
AP	1	27.3 (0.8)	*
AP	2	29.7 (1.8)	51.7 (7.5)
AP	3	44.1(12.0)	56.4(15.2)
AP	4	32.5(15.3)	69.5(24.4)
AP	5	19.2 (2.6)	38.9
AP	6	19.4 (8.2)	59.3 (4.5)
AP	7	30.6 (9.6)	75.0
AP	8	18.7 (1.1)	57.4 (1.6)
AP	9	21.0 (5.8)	60.5(27.0)
AP	10	30.9 (8.2)	65.2 (8.9)
RPP	11	18.0 (2.4)	59.1(19.5)
RPP	12	23.2 (4.7)	68.7(10.3)
RPP	13	34.4 (2.9)	74.1 (4.3)
RPP	14	16.7 (7.2)	65.6 (9.4)
RPP	15	25.9 (3.6)	75.1(11.6)
RPP	16	21.3 (2.2)	82.5(12.5)
RPP	17	26.6 (4.6)	44.0 (2.8)
JP	18	36.9 (1.9)	76.6 (8.4)
JP	19	30.5 (6.0)	69.9 (5.9)
JP	20	23.2 (7.6)	71.6(16.4)

* missing value

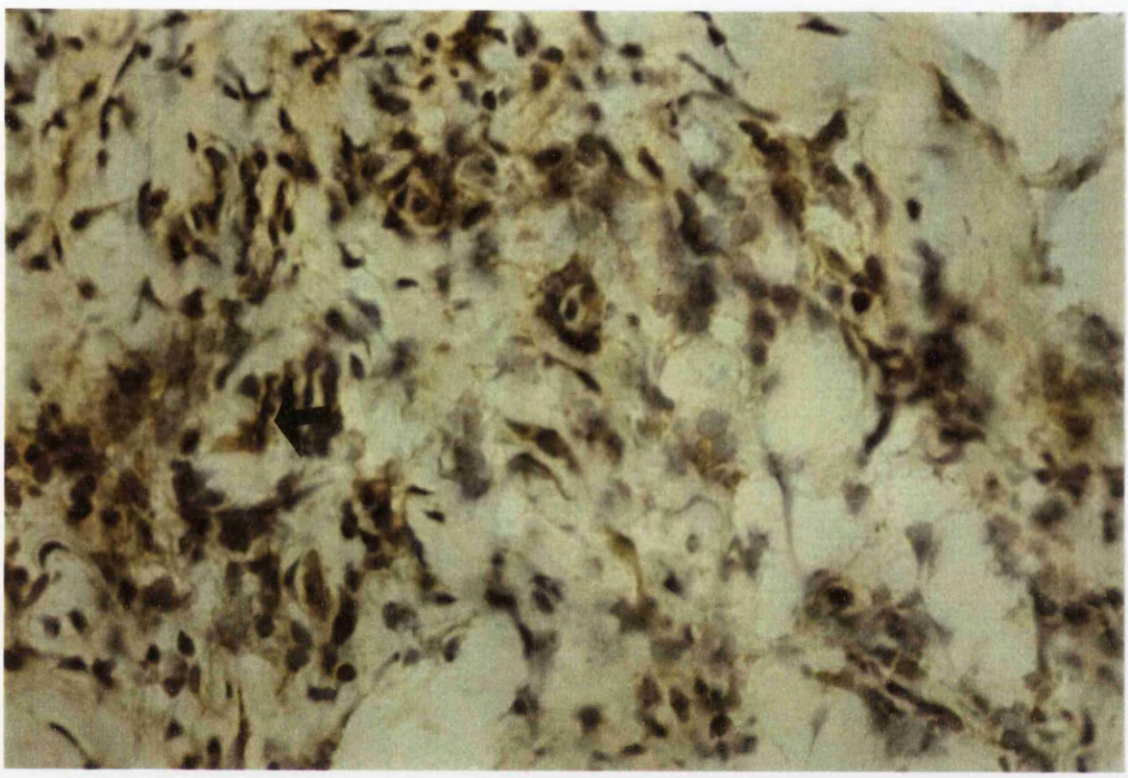

Figure 3.51 Adult periodontitis tissue: ICAM-1^{+} vessels within inflammatory infiltrate (solid arrow) (Immunoperoxidase, mag. x500).

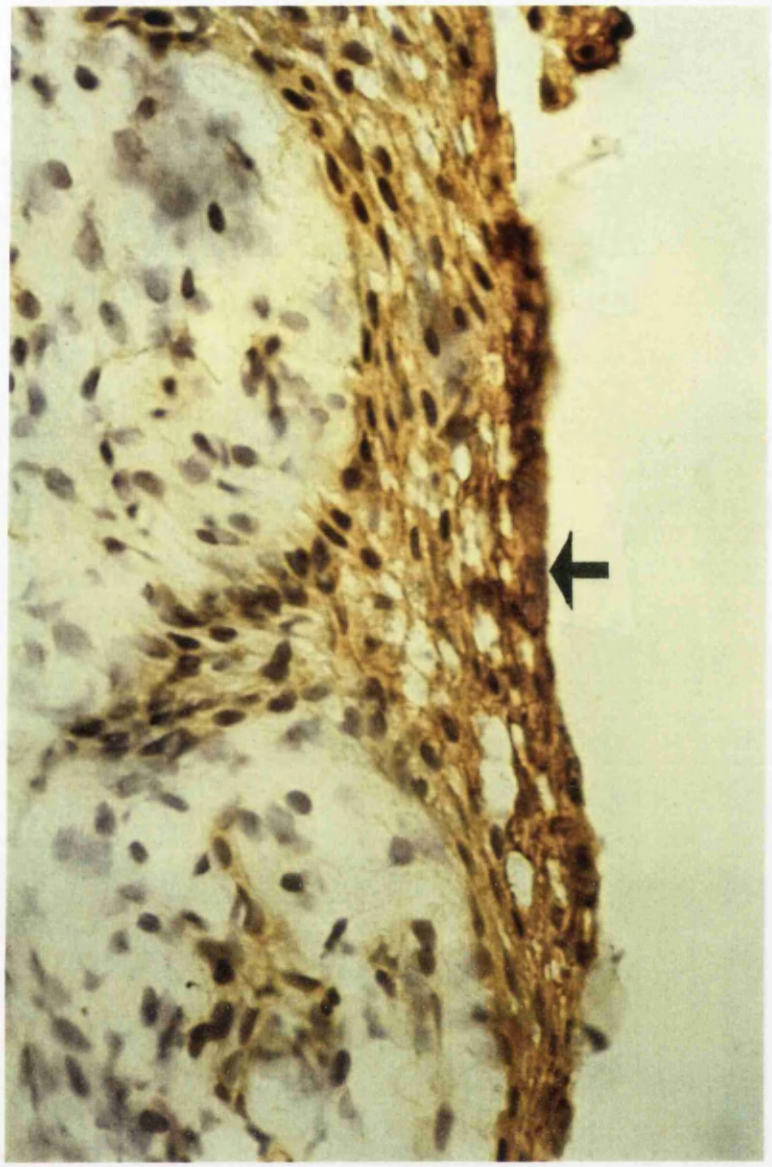

Figure 3.52 Juvenile periodontitis tissue: ICAM-1 gradient, with the most intense staining towards the outer aspect (solid arrow) (Immunoperoxidase, mag. x500).

to surgical variation. Intensity of ICAM-1 staining varied between biopsies but within a biopsy staining intensity of the blood vessels was fairly constant (Table 3.16).

The percentage of ICAM-1 positive vessels per biopsy were also calculated. The values ranged from 38.9 to 75.0% (Table 3.17).

3.3.2 Rapidly progressive periodontitis

3.3.2.1 ELAM-1 expression

ELAM-1 positive vessels were located within areas of cellular infiltration, one biopsy exhibited ELAM-1 keratinocyte staining. Intensity of staining also varied between biopsies but not within biopsies (Table 3.16).

The percentage of ELAM-1 positive vessels ranged from 16.7 to 34.3% (Table 3.17).

3.3.2.2 ICAM-1 expression

Epithelium ICAM-1 staining was seen in most biopsies, with some biopsies exhibiting preferential staining (Fig. 3.53). When epithelium staining was present it exhibited a gradient. Vessel staining within connective tissue was seen located within the cellular infiltrate, if present. Intensity of staining varied between biopsies but not within biopsies (Table 3.16).

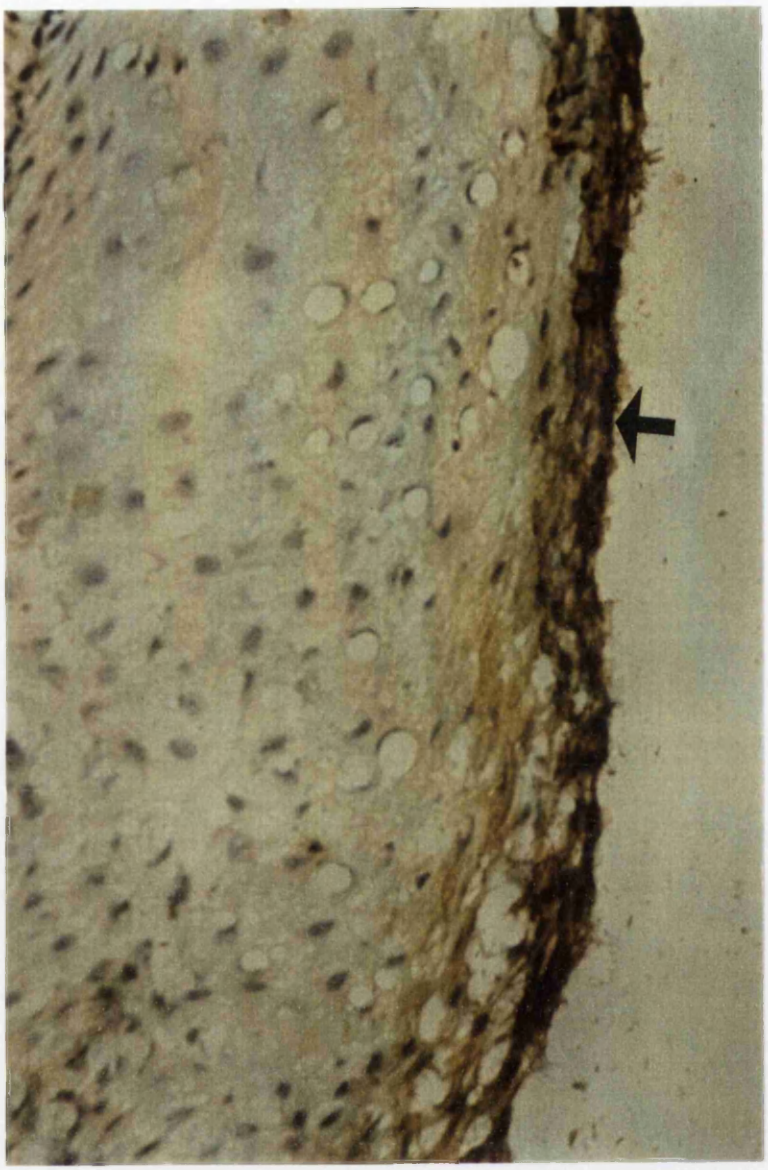

Figure 3.53 Rapidly progressive periodontitis tissue: ICAM-1 staining of the outer layers of the oral epithelium (solid arrow) (Immunoperoxidase, mag. x500).

The percentage of ICAM-1$^+$ vessels were calculated and found to range from 44.0 to 82.5% (Table 3.17).

3.3.3 Juvenile periodontitis

3.3.3.1 ELAM-1 expression

ELAM-1$^+$ vessels were located within cellular infiltrate, if present. Intensity of staining can be seen in Table 3.16.

The percentage of ELAM-1$^+$ vessels varied from 23.2 to 36.9% (Table 3.17).

3.3.3.2 ICAM-1 expression

ICAM-1 positive keratinocytes were demonstrated with connective tissue vessel staining seen within the cellular infiltration. Staining intensity varied between biopsies (Table 3.16).

The percentage of ICAM-1 positive vessels ranged from 69.9 to 76.6% (Table 3.17).

3.4 Gingival crevicular washings

GCW was obtained from a total of 63 sites (section 2.2.4.1). The mean MGI and PD of the sites were 1.87 (±1.28) and 3.19 mm (±2.2) respectively (mean ± standard deviation in parenthesis). Both LF levels (ng/µl GCW) and PMN numbers (PMNs/µl) were determined in each sample. The relationship between GCW LF levels and PMN numbers was examined, the Pearson's correlation was determined

(r=0.531, p<0.001) and the least squares method was used to plot the best fitting line (Fig. 3.54). The association between both GCW LF levels and GCW PMN numbers and the clinical indices was also assessed (Table 3.18). GCW LF levels and PMN numbers correlated positively and significantly with both MGI and PD but LF demonstrated consistently higher correlation coefficients than PMNs with both clinical indices (Table 3.18).

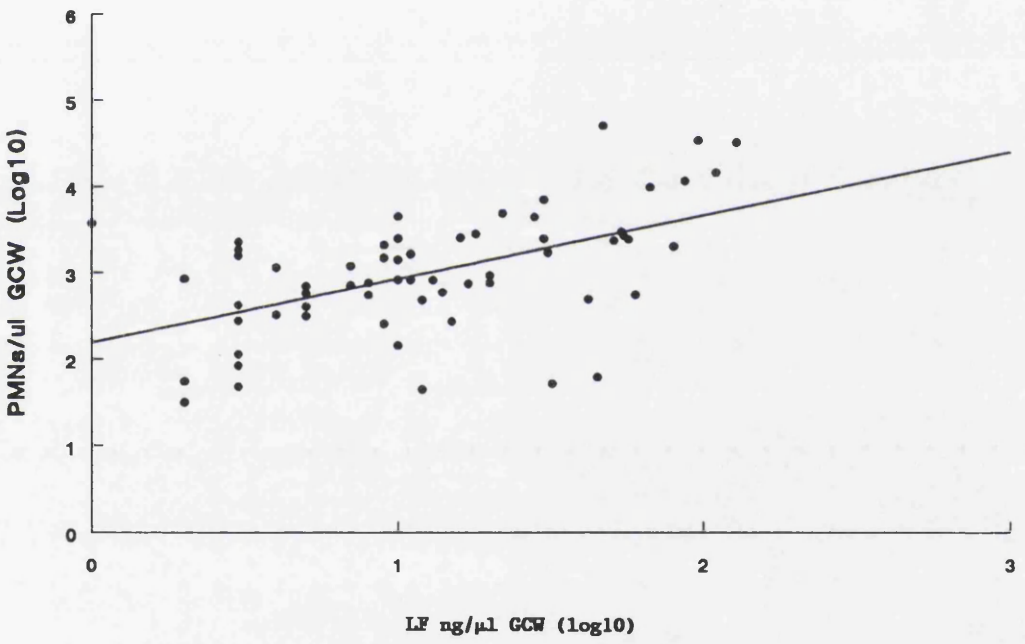

Figure 3.54 PMNs/µl gingival crevicular washing (GCW) as function of GCW lactoferrin levels (ng/µl GCW) in n=63 GCWs. Both variables are transformed to $\log_{10}$. The Pearson correlation coefficient ($r=0.531$, $p<0.001$) was determined and the best fitting line plotted by the least squares method (regression analysis).

Table 3.18 Spearman rank correlation coefficient (r) between clinical indices and GCW LF levels and PMN numbers of n=63 GCWs obtained. Probability levels (p) shown in parenthesis.

Parameter	LF(ng/µl GCW)	PMNs (PMNS/µl GCW)
MGI	r=0.452 (p<0.001)	r=0.279 (p<0.030)
PD	r=0.513 (p<0.001)	r=0.388 (p<0.003)

LF: Lactoferrin; MGI: Modified gingival index;
PD: Pocket depth

Chapter 4

Discussion

4.1 Introduction

The aims of this thesis were, firstly, to study the immune and inflammatory cellular infiltrate during the development of gingival inflammation utilising the experimental gingivitis model; secondly, to examine the effect of cytokines on adhesion molecule expression using gingival organ culture; thirdly, to assess inflammatory changes within diseased periodontal tissue; and finally, to draw conclusions on the interactions and the roles played by the different variables analysed in this thesis.

In order to present this work clearly, the discussion is arranged into sections dealing with particular topics, but interactions between particular areas are also developed.

4.2 Clinical parameters

Clinical parameters of plaque and gingival inflammation increased and peaked after 21-days of oral hygiene abstention with plaque accumulation preceding the rise in gingival inflammation (Fig. 3.1). Within the 10-day experimental gingivitis study minimal changes in the clinical indices were detected (Fig. 3.26). When Löe *et al.* (1965) first described the experimental gingivitis model they pointed out that at least 10 to 15 days of oral hygiene abstention were required in order to observe clinically detectable gingival inflammation and more recently, other investigators have pointed out that histopathological changes within the gingiva may be

occurring even within the clinically healthy situation (Brecx *et al.*, 1987a); and that initial changes within gingiva are not necessarily clinically detectable (Brecx *et al.*, 1987b). Therefore, the 21-day study was used to determine if changes within adhesion molecules and periodontal cellular infiltrate were occurring during clinically detectable inflammation; and then the 10-day study was designed to investigate very early changes in gingival inflammation by concentrating the 4 biopsy and sampling procedures within the first ten days. The 10-day study was to ascertain if any of the changes noted in the 21-day study were seen when inflammation was histologically detectable, albeit not clinically detectable.

4.3 Interleukin-1

IL-1 has been implicated in the pathogenesis of periodontal disease i.e. bone loss (Mochan *et al.*, 1988; Richards & Rutherford, 1990; Stashenko *et al.*, 1991a, 1991b). Studies on GCF have demonstrated that elevated levels of IL-1 are present in disease sites when compared with healthy sites. Some studies indicate that the predominate IL-1 is IL-1α (Kabashima *et al.*, 1990), whereas other studies suggest that the important IL-1 is IL-1β; and therefore have only looked for this cytokine (Hönig *et al.*, 1989; Jandinski *et al.*, 1991; Wilton *et al.*, 1992). One study looked at both IL-1α and IL-1β, and found that more IL-1β was present than IL-1α in sites manifesting active periodontitis (Masada *et al.*, 1990). The GCF IL-1 levels during the 21-day

experimental gingivitis study show an initial peak and then a drop to baseline levels. The early peak in IL-1 levels reported here (section 3.1.1.10) is consistent with the 'burst hypothesis' (Socransky *et al.*, 1984) of periodontal disease progression, and if this dramatic change in IL-1 levels occurs in periodontitis sites it could be an indicator of incipient breakdown at those sites.

Although IL-1 was originally considered to be a product of mononuclear phagocytes, recent evidence suggests that keratinocytes and gingival fibroblasts can also produce IL-1 in response to stimulation by bacterial components (Walsh, Seymour & Powell, 1985; Hanazawa *et al.*, 1985, 1988; Takada *et al.*, 1991). IL-1 is one of the factors known to stimulate bone resorption and collagenase production by fibroblasts, both of which are features of periodontal destruction (Gowen *et al.*, 1983; Postlethwaite *et al.*, 1983). In addition, as changes in IL-1 levels were detected well in advance of clinically recognisable gingival changes, IL-1 may have a role as an early marker of gingival inflammatory changes.

4.4 Langerhans cells

Conflicting data exists on the dynamics of LC numbers during gingival inflammation. Many workers have suggested that as inflammation develops there is an increase in the number of LC present within gingival OE (Newcomb *et al.*, 1982; DiFranco *et al.*, 1985; Newcomb & Powell, 1986a,

1986b). However, a recent cross-sectional study by Hitzig *et al.* (1989) where tissue was categorised according to clinical indices, suggested that during initial inflammation there was an increase in LC numbers, and in severe inflammation this decreased, contradicting previous studies. This work provides an insight into the changes in LC number within the gingival OE as inflammation develops. The results from the 21-day experimental gingivitis study (section 3.1.1.9) support the view of Hitzig *et al.* (1989) that there is initially an increase in LC numbers followed by a decrease as inflammation develops.

Difranco *et al.* (1985), however compared treated and untreated lesions within the same individual using OKT6, S-100 and HLA-DR markers. These workers found that the numbers of LCs present in the inflamed tissue were significantly increased, and that the best marker for LCs was OKT6 which gave a maximum number of LCs in the gingival epithelium of 9.8 LC/mm^2. This is extremely low when compared to the maximum number of LCs reported in the present studies (261 and 389 LC/mm^2; 10-day and 21-day respectively) and when compared to the study of Hitzig *et al.* (1989) (117 LC/mm^2). These comparisons would suggest that the absolute number of LCs found in the Difranco *et al.* (1985) study, was either severely depressed or their detection or enumeration system was not as sensitive as more recent techniques. Ahlfors *et al.* (1985) suggest that the requirement for the induction of hypersensitivity is

>150 cells/mm^2, this requirement was met by both recent studies. Interestingly, Hitzig *et al.* (1989) used thinner sections 6μm, than the 8μm of the present studies, and this plus a more restrictive criteria for LC determination may account for the higher numbers seen between the present studies and theirs. Newcomb *et al.* (1982) employed adenosine triphosphatase to compare LC numbers in gingival tissue obtained after 0, 8 and 21 days of plaque accumulation. These workers found a statistically significant increase in the numbers of LC present as plaque accumulated (day 0 to day 8). However, with the development of gingival inflammation (day 8 to day 21), there was no difference in the number of LCs present. These results contradict the results of the 10-day experimental gingivitis study which indicated no change in the number of LCs as plaque accumulated (section 3.1.2.10); also the 21-day experimental gingivitis study reports a peak in the number of LCs present after 7 days of plaque accumulation (which failed to reach statistical significance), and a statistically significant decrease in CD1$^+$ LCs seen between days 7 and 21 and days 14 and 21 (section 3.1.1.9). Newcomb & Powell, (1986b) have more recently demonstrated that adenosine triphosphatase is not as specific for LCs as the OKT6 monoclonal antibody.

The above studies examined LC numbers in different individuals; diseased compared with healthy individuals or at different time points, sites within the same diseased

individual. Closer evaluation of the data from these studies suggests that the numbers of LCs detected within disease tissue are severely decreased in comparison with the present studies, where inflammation is induced experimentally. This suggests that with long-term periodontal inflammation LC numbers decrease.

In the 10-day experimental gingivitis study the changes in HLA-DR expression on LCs corresponds to the pattern of change seen in CD1a$^+$ LCs (Fig. 3.44); but with the 21-day experimental gingivitis study the number of Class II MHC positive (HLA-DR$^+$) LCs plateaued between day 7 and day 14 and returned to baseline levels by day 21. The number of CD1$^+$ LCs decreased between day 7 and day 14 and this might be explained if the CD1a$^+$ LCs present on day 0 consisted of some HLA-DR$^-$ cells which become HLA-DR$^+$ cells as inflammation developed (Walsh *et al.*, 1988). The HLA-DR specific monoclonal antibody could also be detecting macrophages. Further histochemical analysis however, using the macrophage specific OKM1 monoclonal antibody (Ortho), suggested that macrophages were too infrequent within the epithelium to appreciably effect the numbers of HLA-DR$^+$ cells.

The inflammatory reaction produced in experimental gingivitis results in various cells releasing cytokines and lymphokines (Charon *et al.*, 1982a; Mergenhagen, 1984; O'Neill & Woodson, 1986). The differential effects of

cytokines on LCs surface antigen expression may modulate the subpopulation of LCs present (Walsh *et al.*, 1988; Ishii *et al.*, 1990). Walsh, Seymour & Powell, (1990b) have demonstrated that Class II MHC positive ($HLA\text{-}DR^+$) LCs are required for effective antigen presentation and that this expression is important for gingival LCs to initiate a T lymphocyte response to antigen. It is unclear of what significance the LC number changes reported here are, but a flux of LCs into and out of the gingival epithelium may be occurring analogous to the changes seen in the number of LCs present at the reaction site during contact hypersensitivity (Silberberg-Sinakin *et al.*, 1980). The flux in LCs may demonstrate movement of these antigen presenting cells to lymph nodes and thus demonstrate a typical mucosal immune antigen detecting and responding process operating in the gingiva.

4.5 Periodontal cellular infiltrate

From the experimental gingivitis studies it was found that within clinically 'healthy' gingival tissue all of the cell types studied (PMNs; T-cells; T-cell subsets: $CD4^+$, $CD8^+$, T-memory and T-naive/virgin) were present even when clinical indices approached zero. This suggests that clinically 'healthy' gingiva is not necessarily histologically healthy (Brecx *et al.*, 1987b) and that minimal inflammatory infiltrate i.e. mild inflammation is compatible with health (Brecx *et al.*, 1987a). Two areas of infiltration were apparent, the JE/CT area and the OE/CT

area. The JE/CT area always contained more inflammatory cells than the OE/CT area, suggesting that the immune cells were preferentially located within this area, due to the fact that the gingival sulcus harbours the antigenic source, bacterial plaque, which is the main cause of gingival inflammation (Löe *et al.*, 1965; Listgarten, 1988; Christersson *et al.*, 1991). As inflammation developed all cell types were still detected within the gingival tissues, with some quantitative variations.

The abundance of PMNs within healthy gingiva (Figs. 3.6; 3.7 & Table 3.15) and their preferential location at the JE/CT and JE/SE areas appear to substantiate their postulated role as the primary line of defense within the gingival tissue (Brecx *et al.*, 1987a). As inflammation developed a loss of cells from the JE/SE was seen, suggesting that the cells were being lost from the gingival crevice; we have demonstrated that as plaque accumulates there is a tendency for PMNs to migrate into the gingival crevice (Table 3.11) (Kowashi *et al.*, 1980). Although no statistically significant difference was detected in the number of PMNs in the JE/SE or JE/CT areas at different time points this does not mean that the number of PMNs trafficking through the tissues did not increase during the course of experimental gingivitis. It simply reflects the difficulty of trying to assess the dynamic flow of cells into and out of the tissues by counting those caught in the middle at one particular moment in time. In other words, if

the recruitment of PMNs into the tissues is balanced by their loss into the gingival crevice there may be little change in PMN numbers in the tissues even if there is an increase in the rate of traffic over the 10-days or 21-days of plaque accumulation.

It was found that alkaline phosphatase and peroxidase could not be used successfully to double stain two antigens within the same tissue section, due to the fact that both primary monoclonal antibodies were from the same animal species i.e. mouse anti-human, which resulted in problems with cross-reactivity. We were also using frozen cryostat sections which do not maintain their tissue structure during extended periods of staining; and also alkaline phosphatase was found not to be a good marker for surface antigens, which were of primary interest in this study. Therefore single staining immunoperoxidase was utilised to stain for cell types of interest.

All experimental gingivitis histopathological studies have demonstrated that the infiltrate in the developing lesion is of lymphocytic character, mainly the T-cell type (Seymour *et al.*, 1983b, 1988; Brecx *et al.*, 1987b, 1988a, 1988b), with greater than 6 months of plaque accumulation required for plasma cells to dominate the lesion (Brecx *et al.*, 1988a). With the present 21-day and 10-day experimental gingivitis studies we have demonstrated that the experimental lesion is T-cell and PMN dominated (Figs.

3.6; 3.7; 3.9; 3.10; 3.38 & Table 3.15) (Brecx *et al.*, 1988a, 1988b). T-cells were found in great abundance within all areas of healthy and experimentally inflamed gingival tissue. Analysis of T-lymphocyte subsets within the 21-day experimental gingivitis study demonstrated no variation in T4:T8 ratio (Seymour *et al.*, 1988). Seymour *et al.* (1983a, 1983b) comment on the sparse T-lymphocyte infiltrate within 7 to 10 days of experimental gingivitis. The immunohistochemistry technique employed in the present study is very specific and sensitive and accordingly T cell numbers are found in greater numbers than with previous techniques. $CD3^+$ intraepithelial lymphocytes were also detected within the OE, which also appeared to be $CD4^+$ or $CD8^+$. Interestingly $CD3^+$ cells (pan T-cells) appeared to cluster around the tips of the OE rete pegs and on more specific analysis these cells were noted to comprise both $CD4^+$ and $CD8^+$ cells. Within OE rete pegs LCs can be found which are recognised antigen presenting cells (section 3.1.1.9 and 3.1.2.10). It can be postulated that this clustering of $CD4^+$ or $CD8^+$ T-cells around the tips of the OE rete pegs is essential for LCs to be able to initiate an immune response.

No study to date has looked at the accumulation of T-naive/virgin ($CD45RA^+$) or T-memory ($CD45RO^+$) within experimental gingivitis, though Colasante *et al.* (1992) have demonstrated elevated numbers of T-memory cells in healthy gingiva. The 10-day experimental gingivitis study

demonstrated that within healthy gingival tissue both subsets CD45RO (memory) and CD45RA (naive/virgin) were present (Figs. 3.40 & 3.41 respectively); and that the detectable number of both cell types was elevated within the JE/CT area when compared with the OE/CT area. Interestingly within healthy gingiva, the ratio of T-memory to T-naive/virgin cells (i.e. CD45RO:CD45RA) was elevated, but as inflammation develops the ratio of CD45RO:CD45RA decreased. This suggests that only within clinically 'healthy' gingival tissue does the number of detectable T-memory cells supersedes the number of detectable T-naive/virgin cells. As inflammation developed (i.e. days of plaque accumulation) the number of T-memory cells did not vary within the JE/CT, but rather the number of T-naive/virgin cells decreased. Contradicting previous studies, carried out in different tissue types, where an increase in T-memory cells is noted as inflammation develops (Griffiths & Nickoloff, 1989; Rustin *et al.*, 1990; Gilmore, Benson & Kelly, 1991; Picker *et al.*, 1991; Shimizu *et al.*, 1991). It is possible that within the experimentally inflamed gingival tissue, loss of activated T-memory cells into the gingival crevice may be occurring because T-memory cells are better adapted to respond to antigen (Mackay, 1991). Also, as no change is seen in the number of T-memory cells, it is feasible that although a deficit is occurring due to the flux of T-memory cells into the crevice, it is being filled by the naive cells maturing and switching to the memory cell phenotype (Wallace &

Beverley, 1990; Gilmore *et al.*, 1991).

Quantitative and qualitative changes within periodontal cellular infiltrate during experimental gingivitis was occurring. But the changes were not apparent when one time point was compared to another. It must be remembered that the immune response is a dynamic process, and that even with sequential biopsies it is difficult to determine exactly the specific changes occurring.

4.6 Adhesion molecules

The studies presented in this thesis demonstrate the presence of the adhesion molecules, ELAM-1, ICAM-1 and VCAM-1, in clinically healthy, experimentally inflamed and diseased gingival tissue. Whereas previous studies have shown that normal 'uninflamed' tissue is negative for ELAM-1 (Cotran *et al.*, 1986; Bevilacqua *et al.*, 1987; Munro *et al.*, 1989; Munro, Pober & Cotran, 1991; Rohde *et al.*, 1992) and expresses low levels of ICAM-1 (Lewis *et al.*, 1989; Majewski *et al.*, 1991), with minimal VCAM-1 expression (Norris *et al.*, 1991; Groves *et al.*, 1992).

In vitro studies have shown that ELAM-1 acts as a highly specific endothelial cell ligand for the binding of peripheral blood PMNs (Bevilacqua *et al.*, 1987, 1989; Rohde *et al.*, 1992), especially ones rich in sialylated Lewis X antigen (Fukuda *et al.*, 1984; Symington *et al.*, 1985; Lowe *et al.*, 1990; Phillips *et al.*, 1990; Walz *et al.*, 1990) and

for T-memory cells (Picker *et al.*, 1991, Shimizu *et al.*, 1991). *In vivo* studies have confirmed a strong correlation between ELAM-1 expression on blood vessels and the accumulation of PMNs and memory-T cells in the tissues during inflammatory responses (Munro *et al.*, 1989, 1991; Norris *et al.*, 1991; Picker *et al.*, 1991, Shimizu *et al.*, 1991). The intensity of ELAM-1 staining of the blood vessels was high at each time point with the strongest expression of ELAM-1 occurring in the same areas (JE and JE/CT) where PMN and T-memory cells were located in greatest abundance.

ICAM-1 has been shown to play a role in the adhesion of PMNs and T-cells to endothelial cells and it is also important in facilitating the trans-endothelial cell migration of leucocytes (Dustin *et al.*, 1986; Rothlein *et al.*, 1986; Lewis *et al.*, 1989; Majewski *et al.*, 1991), especially cells of the memory phenotype (Griffiths & Nickoloff, 1989; Buckle & Hogg, 1990) and cells expressing LFA-1 (CD11a) (Dustin *et al.*, 1986). ICAM-1 expression on the blood vessels changed very little in relation to its distribution throughout the 10 and 21 days and this was reflected in the T-cell numbers not changing within the different tissue compartments (Figs. 3.9 & 3.10).

Within gingival tissue the percentage of biopsies with greater JE/CT than OE/CT ICAM-1 staining was low (range 0 to 40%) (values calculated from Table 3.4) i.e. staining

within JE/CT and OE/CT was similar. Cells within gingival tissue expressing the ICAM-1 ligand CD11a, were elevated within JE/CT and interestingly within OE/CT as well (Fig 3.34). This elevated number of $CD11a^+$ cells within the OE/CT and the unbiased expression of ICAM-1 suggests that ICAM-1 may be important in targeting $CD11a^+$ cell migration to OE/CT, for antigen presentation by gingival LCs and initiation of an immune response.

It was noted that junctional epithelium, and to a lesser extent sulcular epithelium, remained strongly positive for ICAM-1 throughout the 10 and 21 days of experimental gingivitis. At no point was ICAM-1 positivity of the adjacent oral epithelium noted and the transition from ICAM-1 positive junctional and sulcular epithelium to oral epithelium was always quite distinct and marked (Fig. 3.3). The staining showed a gradient of intensity with the most intense staining at the crevicular aspect and the weakest on the connective tissue aspect (Fig. 3.5). The intensity of the ICAM-1 staining at JE is greater than is often seen in other epithelial tissues even in the presence of an immune mediated inflammatory response or direct cytokine stimulation. Taken together these observations suggest that junctional epithelium may be specially adapted to constitutively express ICAM-1. Since ICAM-1 expression on keratinocytes facilitates leucocyte-keratinocyte adhesion and transmigration (Dustin & Springer, 1988) this adaption may help to explain the special ability of junctional

epithelium to permit leucocyte traffic into the gingival crevice. Unlike endothelial cells, keratinocytes do not normally express ICAM-1, however, keratinocyte ICAM-1 expression can be induced both *in vivo* and *in vitro* by the action of interferon-γ but not by interleukin-1 or LPS (Barker *et al.*, 1989; Krutmann *et al.*, 1990). Epithelial cell ICAM-1 expression is therefore only observed during immune mediated inflammatory processes such as delayed type hypersensitivity (Norris *et al.*, 1991) and mucosal lichen planus (Walsh *et al.*, 1990a). Even then epithelial cell ICAM-1 staining is not usually very strong and is restricted to areas with the most intense lymphocytic infiltrates. It was therefore unexpected to find very intense ICAM-1 staining of JE even in clinically 'healthy' gingivae. During the development of the experimental gingivitis there was no significant alteration in the intensity or pattern of staining. These observations strongly suggest that JE may be adapted to express ICAM-1 constitutively even in the absence of interferon stimulation. In theory IL-1 or LPS in the gingival crevice could upregulate ICAM-1 expression and this may explain the gradient effect seen in the JE, with the most intense staining seen on the outer aspect and may reflect the fact that JE is the site of extensive PMN migration in both health and disease (Attström & Egelberg, 1970; Schiött & Löe, 1970; Scully & Challacombe, 1979).

VCAM-1 expression within gingival tissue did not change in its intensity when JE/CT area was compared with OE/CT area, or when biopsies at different time points were compared. VCAM-1 expression has been linked with T-cell migration (Osborn *et al.*, 1989; Graber *et al.*, 1990), but not PMNs (Osborn *et al.*, 1989). The ligand for VCAM-1 has been elucidated as the VLA-4 integrin (Elices *et al.*, 1990).

Kinetical expression of the adhesion molecules has been studied *in vitro* and *in vivo*, and has been shown to differ. *In vitro*, ELAM-1 expression can be detected by 4-6 hours and returns to baseline by 24 hours (Bevilacqua *et al.*, 1987, 1989; Messadi *et al.*, 1987). *In vivo* ELAM-1 expression is detected at 6 hours, maximal at 24-72 hours if immune-mediated, or returns to baseline by 24 hours if non-immune mediated (Norris *et al.*, 1991). ICAM-1 expression is detectable at 10-24 hours and is maintained for 72 hours (Pober *et al.*, 1986; Wellicome *et al.*, 1990). In contrast, VCAM-1 is detected at 2 hours, maximal at 6-10 hours and returns to baseline by 24 hours if stimulated by IL-1 or LPS, and is maintained if stimulated by TNF (Thornhill & Haskard, 1990; Wellicome *et al.*, 1990). It appears that ELAM-1 expression increases initially followed by VCAM-1 and finally by ICAM-1. The fact that the adhesion molecules ELAM-1, ICAM-1 and VCAM-1 are detected throughout the 10 and 21 days of experimentally induced inflammation suggests one of two possibilities, either the blood vessels in gingiva are functionally specialised and

constitutively express ELAM-1 and VCAM-1 plus high levels of ICAM-1 to facilitate leucocyte traffic into the gingival crevice or the gingival vessels are permanently activated even in 'health' due perhaps to the constant ingress of bacterial products such as LPS across the junctional epithelium. Further support for the idea that gingival post-capillary venules may be specialised to express ELAM-1 and VCAM-1 constitutively, is provided by the fact that they do not appear to exhibit the normal modulation of ELAM-1 and VCAM-1 expression seen in other situations (Munro *et al.*, 1989, 1991; Thornhill & Haskard, 1990; Wellicome *et al.*, 1990; Norris *et al.*, 1991).

When the relationship between JE/CT and OE/CT staining was compared for ELAM-1 and ICAM-1, it was found that ELAM-1 staining correlated better at 10-days than at 21-days ($r=0.763$ and $r=0.528$, respectively); whereas ICAM-1 showed the opposite effect, with 21-days correlating better than 10-days ($r=0.751$ and $r=0.518$, respectively). This suggests that ELAM-1 is important in early inflammation and that ICAM-1 becomes more important as inflammation develops. This would correlate with our understanding of the sequence of inflammatory cell infiltration in acute inflammatory lesions i.e. PMNs first followed by the chronic inflammatory cells, macrophages and lymphocytes.

Cytokines increase the expression of the adhesion molecules (Pober *et al.*, 1986; Wellicome *et al.*, 1990; Norris *et al.*, 1991). Our organ culture studies have demonstrated that cytokines have differing effects on gingival tissue, depending upon its state of activation i.e. depending which block of tissue is used (Figs. 3.45 - 3.48). Again suggesting that gingival tissue is an unusual inflammatory situation. In addition, the 21-day experimental gingivitis study indicated IL-1's presence and its dynamics in the crevice which may be involved in upregulating adhesion molecules.

The appearance of high endothelial venules (HEV) during the development of experimental gingivitis is one indication that gingival blood vessels may become altered to support leucocyte traffic (Wynne *et al.*, 1988). HEV were first described in lymphoid tissue and represent specialised postcapillary venules across which large numbers of lymphocytes traffic during their recirculation from the blood into lymphoid tissue and back to blood via the lymphatics. Instead of having the normal flat morphology of EC they have a much more cuboidal appearance. HEVs have also been observed in several long standing chronic inflammatory situations in which large numbers of lymphocytes accumulate (Nightingale & Hurley, 1978; Freemont & Ford, 1985). In lymphoid tissue, HEV express organ specific 'addressin' molecules that enable subpopulations of lymphocytes, including B cells, and

plasma cells, to recirculate through specific lymphoid organs (Jalkanen *et al.*, 1987). The appearance of HEV in inflammatory periodontal lesions may therefore mark the development of a lesion through which specific subpopulations of lymphocytes are recirculating in large numbers and also a lesion to which B cells and plasma cells are able to home (Jutila *et al.*, 1989).

4.7 Conclusion

It could be hypothesised that within clinically 'healthy' tissue mild inflammation is always present and that the gingiva is never really healthy, perhaps due to the constant presence of the antigenic stimulus - bacterial plaque and the fact that junctional epithelial cells are loosely bound and permit passage of molecules between cells. The continued onslaught of antigenic challenge keeps the gingiva in a mild state of inflammation where the immune cells are playing an immunosurveillance role keeping the gingiva clinically 'healthy'. With the accumulation of plaque, as in the experimental gingivitis model, changes start to occur histologically at first and then clinically visible changes appear. Histologically the size of the inflammatory infiltrate starts to increase, at different rates within different individuals (Table 3.2 and 3.9) (Löe *et al.*, 1965) and there is an early peak in IL-1 levels. PMNs within healthy gingiva are found at the crevicular/tooth aspect (section 3.1.1.4) (Brecx *et al.*, 1987a) and as inflammation develops, loss of PMNs from the

gingival tissue is seen (section 3.1.1.4), with PMNs migrating into the gingival crevice (Sigusch *et al.*, 1992). Simultaneously changes occur in LC numbers within the first weeks of gingival inflammation. This may reflect movement in and out of gingival epithelium and suggests that these LCs are active in antigen presentation even at this early stage of inflammation. Migration of gingival LCs into the lymphoid system results in stimulation of lymphocytes and initiation of an immune response. This flux of LCs may represent an important early "immune triggering" event in the gingival immune response to plaque. ECs play an important role in orchestrating inflammatory and immune events. Central to this is the key role played by cytokines and 'adhesion molecules' in regulating leucocyte-EC adhesion, the first step in leucocyte migration. The preliminary evidence presented in this thesis suggests that the gingival tissues may be specifically adapted, with regard to their expression of adhesion molecules, to facilitate the migration of PMNs and other cell types into the gingiva and into the crevice as part of the normal defence of the periodontium. Immunological cellular infiltration is seen within the tissues as gingival inflammation develops. The spatial and temporal changes in the composition of the cellular infiltrate illustrates the difficulty in interpretation using sequential biopsies.

The importance of these endothelial and leucocyte adhesion mechanisms in protecting against periodontal disease is

highlighted by the rapid and severe periodontitis that characterises LAD (leucocyte adhesion deficiency) (Anderson & Springer, 1987; Page *et al.*, 1987; Waldrop *et al.*, 1987). It is important that the kinetics of adhesion molecule expression and the dynamics of leucocyte trafficking are studied in greater detail in order to ascertain if defects in adhesion molecule expression play a part in determining an individuals susceptibility to periodontal disease.

4.8 Future research

These studies have demonstrated the presence of adhesion molecules and cellular infiltrate within gingival tissue and have also suggested that changes in Langerhans cell numbers within oral epithelium are occurring early on in gingival inflammation. Changes within gingival cellular infiltrate may be occurring but as we are looking at a dynamic process in a cross-sectional manner, it is difficult to visualise the true changes occurring. Future research should be aimed at examining the flux of cells into the crevice, into the connective tissue and/or arriving at the blood vessels, using time-lapse video analysis. Also, studies need to be performed to obtain a more specific understanding of the role of these adhesion molecules in chronic inflammatory periodontal disease; and also to examine the manner in which adhesion molecules control the accumulation of lymphocytes into chronic inflammatory tissue.

Studies so far have demonstrated that blood vessels within the gingiva are specialised, future research should focus on trying to elucidate the effect of various cytokines on the expression of their cellular adhesion molecules. Do the cytokines effect gingival tissue in the classical way they act on other types of tissue? or do gingival vessels express unique kinetics? Cytokines within GCF also need to be elucidated to ascertain whether a single cytokine or a combination of cytokines and/or LPS is responsible for the gradient ICAM-1 effect seen in junctional epithelium. The answers to these questions may further clarify the role of adhesion molecule expression and cellular infiltrate within gingival tissue.

References

Afar, B., Engel, D. & Clark, E.A. (1992) Activated lymphocyte subsets in adult periodontitis. *Journal of Periodontal Research*, **27**, 126-133.

Ahlfors, E.E., Larsson, P.A. & Bergstresser, P.R. (1985) Langerhans cell surface densities in rat oral mucosa and human buccal mucosa. *Journal of Oral Pathology*, **14**, 390-397.

Ainamo, J. & Löe, H. (1966) Anatomical characteristics of gingiva. A clinical and microscopic study of the free and attached gingiva. *Journal of Periodontology*, **37**, 5-13.

Akbar, A.N., Terry, L., Timms, A., Beverley, P.C.L. & Janossy, G. (1988) Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *The Journal of Immunology*, **140**, 2171-2178.

Allison, F.Jr., Smith, M.R. & Wood, W.B.Jr. (1955) Studies on the pathogenesis of acute inflammation. I The inflammatory reaction to thermal injury as observed in the rabbit ear chamber. *Journal of Experimental Medicine*, **102**, 655-676.

Altman, L.C., Baker, C., Fleckman, P., Luchtel, D. & Oda, D. (1992) Neutrophil-mediated damage to human gingival epithelial cells. *Journal of Periodontal Research*, **27**, 70-79.

Amer, A., Singh, G., Darke, G. & Dolby, A.E. (1990) Spontaneous lymphocyte proliferation in severe periodontal disease: role of T and B cells. *Journal of Oral Pathology and Medicine*, **19**, 49-52.

Ammons, W.F., Schectman, L.R. & Page, R.C. (1972) Host tissue response in chronic periodontal disease I. The normal periodontium and clinical manifestations of dental and periodontal disease in the marmoset. *Journal of Periodontal Research*, **7**, 131-143.

Anderson, D.C. & Springer, T.A. (1987) Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annual Reviews in Medicine*, **38**, 175-194.

Arnim, S.S. & Hagerman, D.A. (1953) The connective tissue fibers of the marginal gingiva. *Journal of the American Dental Association*, **47**, 271-281.

Attström, R. (1970) Presence of leukocytes in crevices of healthy and chronically inflamed gingivae *Journal of Periodontal Research*, **5**, 42-47.

Attström, R. (1971) Studies on neutrophil polymorphonuclear leukocytes at the dento-gingival junction in gingival health and disease. *Journal of Periodontal Research*, **6**, supplement 8.

Attström, R. & Egelberg, J. (1970) Emigration of blood neutrophils and monocytes into the gingival crevices. *Journal of Periodontal Research*, **5**, 48-55.

Attström, R. & Egelberg, J. (1971) Presence of leukocytes within the gingival crevices during development of gingivitis in dogs. *Journal of Periodontal Research*, **6**, 110-114.

Attström, R., Graf-De Beer, M. & Schroeder, H.E. (1975) Clinical and histologic characteristics of normal gingiva in dogs. *Journal of Periodontal Research*, **10**, 115-127.

Attström, R. & Schroeder, H.E. (1979) Effect of experimental neutropenia on initial gingivitis in dogs. *Scandinavian Journal of Dental Research*, **87**, 7-23.

Attström, R., Tynelius-Bratthall, G. & Egelberg, J. (1971) Effect of experimental leukopenia on chronic gingival inflammation in dogs. II. Induction of leukopenia by heterologous anti-neutrophil serum. *Journal of Periodontal Research*, **6**, 200-210.

Awbrey, J.J. & Hibbard, E.D. (1973) Abbreviated case report. Congenital agranulocytosis. *Oral Surgery, Oral Medicine and Oral Pathology*, **35**, 526-530.

Badersten, A., Nilveus, R. & Egelberg, J. (1985) Effect of nonsurgical periodontal therapy. V. Patterns of probing attachment loss in non-responding sites. *Journal of Clinical Periodontology*, **12**, 270-282.

Baehni, P.C., Payot, P., Tsai, C.-C. & Cimasoni, G. (1983) Periodontal status associated with chronic neutropenia. *Journal of Clinical Periodontology*, **10**, 222-230.

Baelum, V., Fejerskov, O. & Dabelsteen, E. (1989) Langerhans cells in oral epithelium of chronically inflamed human gingivae. *Journal of Periodontal Research*, **24**, 127-136.

Baer, P.N. (1971) The case for periodontosis as a clinical entity. *Journal of Periodontology*, **42**, 516-520.

Barker, J.N.W.N., Allen, M.H. & MacDonald, D.M. (1989) The effect of *in vivo* interferon-gamma on the distribution of LFA-1 and ICAM-1 in normal human skin. *Journal of Investigative Dermatology*, **93**, 439-442.

Bartold, P.M. (1988) The effect of interleukin-1β on hyaluronic acid synthesized by adult human gingival fibroblasts *in vitro*. *Journal of Periodontal Research*, **23**, 139-147.

Barton, R.W., Rothlein, R., Ksiazek, J. & Kennedy, C. (1989) The effect of anti-intercellular adhesion molecule-1 on phorbol-ester-induced rabbit lung inflammation. *The Journal of Immunology*, **143**, 1278-1282.

Becker, S. (1985) Interferon-gamma accelerates immune proliferation via its effect on monocyte HLA-DR expression. *Cellular Immunology*, **91**, 301-307.

Benedek-Spät, E., Di Felice, R., Andersen, E. & Cimasoni, G. (1991) *In vitro* release of elastase from human blood and gingival crevicular neutrophils. *Archives of Oral Biology*, **36**, 507-510.

Bernstein, A.B., Preisig, E. & Schroeder, H.E. (1988) Formation of a new fibrous attachment to human dental roots. A new *in vitro* model for studying periodontal regeneration. *Cell and Tissue Research*, **254**, 659-670.

Bertolini, D.R., Nedwin, G.E., Bringman, T.S., Smith, D.D. & Mundy, G.R. (1986) Stimulation of bone resorption and inhibition of bone formation *in vitro* by human tumour necrosis factors. *Nature*, **319**, 516-518.

Bevilacqua, M.P., Pober, J.S., Mendrick, D.L., Cotran, R.S. & Gimbrone, M.A.Jr. (1987) Identification of an inducible endothelial-leukocyte adhesion molecule. *Proceedings of the National Academy of Sciences of the U.S.A.*, **84**, 9238-9242.

Bevilacqua, M.P., Pober, J.S., Wheeler, M.E., Cotran, R.S. & Gimbrone, M.A.Jr. (1985) Interleukin 1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes and related leukocyte cell lines. *Journal of Clinical Investigations*, **76**, 2003-2011.

Bevilacqua, M.P., Stengelin, S., Gimbrone, M.A.Jr. & Seed, B. (1989) Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science*, **243**, 1160-1165.

Bloom, B.R. & Bennet, B. (1966) Mechanism of a reaction *in vitro* associated with delayed-type hypersensitivity. *Science*, **153**, 80-82.

Bordin, S., Page, R.C. & Narayanan, A.S. (1984) Heterogeneity of normal human diploid fibroblasts: Isolation and characterization of one phenotype. *Science*, **223**, 171-173.

Boughman, J.A., Beaty, T.H., Yang, P., Goodman, S.B., Wooten, R.K. & Suzuki, J.B. (1988) Problem in genetic model testing in early onset periodontitis. *Journal of Periodontology*, **59**, 332-337.

Bral, M.M. & Stahl, S.S. (1977) Keratinizing potential of human crevicular epithelium. *Journal of Periodontology*, **48**, 381-387.

Brecx, M.C., Gautschi, M., Gehr, P. & Lang, N.P. (1987a) Variability of histologic criteria in clinically healthy human gingiva. *Journal of Periodontal Research*, **22**, 468-472.

Brecx, M.C., Fröhlicher, I., Gehr, P. & Lang, N.P. (1988a) Stereological observations on long-term experimental gingivitis in man. *Journal of Clinical Periodontology*, **15**, 621-627.

Brecx, M.C., Lehmann, B., Siegwart, C.M., Gehr, P. & Lang, N.P. (1988b) Observations on the initial stages of healing following human experimental gingivitis. A clinical and morphometric study. *Journal of Clinical Periodontology*, **15**, 123-129.

Brecx, M.C., Schlegel, K., Gehr, P. & Lang, N.P. (1987b) Comparison between histological and clinical parameters during human experimental gingivitis. *Journal of Periodontal Research*, **22**, 50-57.

Brill, N. (1959) Effect of chewing on flow of tissue fluid into gingival pockets. *Acta Odontologica Scandinavica*, **17**, 277-284.

Brill, N. & Brönnestam, R. (1960) Immunoelectrophoretic study of tissue fluid from gingival pockets. *Acta Odontologica Scandinavica*, **18**, 95-100.

Briscoe, D.M., Schoen, F.J., Rice, G.E., Bevilacqua, M.P., Ganz, P. & Pober, J.S. (1991) Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation*, **51**, 537-539.

Buckle, A.-M. & Hogg, N. (1990) Human memory T cells express intercellular adhesion molecule-1 which can be increased by interleukin 2 and interferon-γ. *European Journal of Immunology*, **20**, 337-341.

Burmeister, J.A., Best, A.M., Palcanis, K.G., Caine, F.A. & Ranney, R.R. (1984) Localized juvenile periodontitis and generalized severe periodontitis: clinical findings. *Journal of Clinical Periodontology*, **11**, 181-192.

Byrne, J.A., Butler, J.L. & Cooper, M.D. (1988) Differential activation requirements for virgin and memory T cells. *The Journal of Immunology*, **141**, 3249-3257.

Caffesse, R.G., Karring, T. & Nasjleti, C.E. (1977) Keratinizing potential of sulcular epithelium. *Journal of Periodontology*, **48**, 140-146.

Caffesse, R.G., Kornman, K.S. & Nasjleti, C.E. (1980) The effect of intensive antibacterial therapy on the sulcular environment in monkeys. II. Inflammation, mitotic activity and keratinization of the sulcular epithelium. *Journal of Periodontology*, **5**, 155-161.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. & Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumours. *Proceedings of the National Academy of Sciences of the U.S.A.*, **72**, 3666-3670.

Cattoni, M. (1951) Lymphocytes in the epithelium of healthy gingiva. *Journal of Dental Research*, **30**, 627-637.

Caughman, S.W., Lian-Jie, L. & Degitz, K. (1990) Characterization and functional analysis of interferon-γ-induced intercellular adhesion molecule-1 expression in human keratinocytes and A-431 cells. *Journal of Investigative Dermatology*, **94**, 22S-26S.

Cavender, D.E., Haskard, D.O., Joseph, B. & Ziff, M. (1986) Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *The Journal of Immunology*, **136**, 203-207.

Cavender, D.E., Haskard, D.O., Maliakkal, D. & Ziff, M. (1988) Separation and characterization of human T lymphocytes with varying adhesiveness for endothelial cells. *Cellular Immunology*, **117**, 111-126.

Charon, J.A., Luger, T.A., Mergenhagen, S.E. & Oppenheim, J.J. (1982a) Increased thymocyte-activating factor in human gingival fluid during gingival inflammation. *Infection and Immunity*, **38**, 1190-1195.

Charon, J.A., Metzger, Z., Hoffeld, J.T., Oliver, C., Gallin, J.I. & Mergenhagen, S.E. (1982b) An *in vitro* study of neutrophils obtained from normal gingival sulcus. *Journal of Periodontal Research*, **17**, 614-625.

Christersson, L.A., Zambon, J.J. & Genco, R.J. (1991) Dental bacterial plaques. Nature and role in periodontal disease. *Journal of Clinical Periodontology*, **18**, 441-446.

Cianciola, L.J., Genco, R.J., Patters, M.R., McKenna, J. & van Oss, C.J. (1977) Defective polymorphonuclear leukocyte function in a human periodontal disease. *Nature*, **265**, 445-477.

Cimasoni, G. (1983) *Crevicular fluid updated*. 2nd edition, Basel: Karger.

Cimasoni, G. & Giannopoulou, C. (1988) Can crevicular fluid component analysis assist in diagnosis and monitoring periodontal breakdown? *Periodontology Today. International Congress, Zürich*, 260-270.

Clement, L.T., Yamashita, N. & Martin, A.M. (1988) The functionally distinct subpopulations of human $CD4^+$ helper/inducer T Lymphocytes defined by anti-CD45R antibodies derive sequentially from a differentiation pathway that is regulated by activation-dependent post-thymic differentiation. *The Journal of Immunology*, **141**, 1464-1470.

Cohen, B. (1959) Morphological factors in the pathogenesis of periodontal disease. *British Dental Journal*, **107**, 31-39.

Cohen, D.W. & Morris, A.L. (1961) Periodontal manifestations of cyclic neutropenia. *Journal of Periodontology*, **32**, 159-168.

Cohen, S. (1986) Physiologic and pathologic manifestations of lymphokine action. *Human Pathology*, **17**, 112-121.

Cohen, S., Ward, P.A., Yoshida, T. & Burek, C.L. (1973) Biologic activity of extracts of delayed hypersensitivity skin reactions sites. *Cellular Immunology*, **9**, 363-376.

Colasante, A., Rosini, S., Piattelli, A., Artese, L., Aiello, F.B. & Musiani, P. (1992) Distribution and phenotype of immune cells in normal human gingiva: active immune response versus unresponsiveness. *Journal of Oral Pathology and Medicine*, **21**, 12-16.

Cotran, R.S., Gimbrone, M.A.Jr, Bevilacqua, M.P., Mendrick, D.L. & Pober, J.S. (1986) Induction and detection of a human endothelial activation antigen *in vivo*. *Journal of Experimental Medicine*, **164**, 661-666.

Crawford, J.M. (1992) Distribution of ICAM-1, LFA-3 and HLA-DR in healthy and diseased gingival tissues. *Journal of Periodontal Research*, **27**, 291-298.

Crawford, J.M. & Hopp, B. (1990) Junctional epithelium expresses the intercellular adhesion molecule ICAM-1. *Journal of Periodontal Research*, **25**, 254-256.

Cruchley, A.T., Williams, D.M., Farthing, P.M., Lesch, C.A. & Squire, C.A. (1989) Regional variation in Langerhans cell distribution and density in normal human oral mucosa determined using monoclonal antibodies against CD1, HLADR, HLADQ and HLADP. *Journal of Oral Pathology and Medicine*, **18**, 510-516.

Cui, Y.C., Tai, P.C., Gatter, K.C., Mason, D.Y. & Spry, C.J. (1983) A vascular endothelial cell antigen with restricted distribution in human foetal, adult and malignant tissues. *Immunology*, **49**, 183-189.

Daly, C.G., Clancy, R.L. & Cripps, A.W. (1983a) Lymphocytes from chronically inflamed human gingiva I. Cell recovery and characterization *in vitro*. *Journal of Periodontal Research*, **18**, 67-74.

Daly, C.G., Cripps, A.W. & Clancy, R.L. (1983b) Lymphocytes from chronically inflamed human gingiva II. Immunoglobulin production *in vitro*. *Journal of Periodontal Research*, **18**, 132-138.

Damle, N.K., Childs, A.L. & Doyle, L.V. (1987) Immunoregulatory T lymphocytes in man. Soluble antigen-specific suppressor-inducer T Lymphocytes are derived from the $CD4^+$ $CD45R^-$ $p80^+$ subpopulation. *The Journal of Immunology*, **139**, 1501-1508.

Damle, N.K. & Doyle, L.V. (1990) Ability of human T lymphocytes to adhere to vascular endothelial cells and to augment endothelial permeability to macromolecules is linked to their state of post-thymic maturation. *The Journal of Immunology*, **144**, 1233-1240.

Daniels, T.E. (1984) Human mucosal Langerhans cells: Postmortem identification of regional variations in oral mucosa. *Journal of Investigative Dermatology*, **82**, 21-24.

David, J.R. (1966) Delayed hypersensitivity *in vitro*: Its mediation by-cell free substances formed by lymphoid cell-antigen interaction. *Proceedings of the National Academy of Sciences of the U.S.A.*, **56**, 72-77.

Deasy, M.J., Vogel, R.I., Macedo-Sobrinho, B., Gertzman, G. & Simon, B. (1980). Familial benign chronic neutropenia associated with periodontal disease. A case report. *Journal of Periodontology*, **51**, 206-210.

De Lean, A., Munson, P.J. & Rodbard, D. (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose response curves. *American Journal of Physiology E Endocrinology, Metabolism and Gastrointestinal Physiology*, **235**, E97-E102.

Detmar, M., Tenorio, S., Hettmannsperger, U., Ruszczak, Z. & Orfanos, C.E. (1992) Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells *in vitro*. *Journal of Investigative Dermatology*, **98**, 147-153.

Dewhirst, F.E., Stashenko, P.P., Mole, J.E. & Tsurumachi, T. (1985) Purification and partial sequence of human osteoclast-activating factor: Identity with interleukin 1β. *The Journal of Immunology*, **135**, 2562-2568.

Difranco, C.F., Toto, P.D., Rowden, G., Gargiulo, A.W., Keene, J.J.Jr. & Connelly, E. (1985) Identification of Langerhans cells in human gingival epithelium. *Journal of Periodontology*, **56**, 48-54.

Dinarello, C.A. (1988) Biology of interleukin 1. *FASEB Journal*, **2**, 108-115.

Doukas, J. & Pober, J.S. (1990) IFN-γ enhances endothelial activation induced by tumor necrosis factor but not IL-1. *The Journal of Immunology*, **145**, 1727-1733.

Dumonde, D.C., Wolstencroft, R.A., Panayi, G.S., Matthew, M., Morley, J. & Howson, W.T. (1969) "Lymphokines": non-antibody mediators of cellular immunity generated by lymphocyte activation. *Nature*, **224**, 38-42.

Dustin, M.L., Singer, K.H., Tuck, D.T. & Springer, T.A. (1988) Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by interferon γ and is mediated by intercellular adhesion molecule 1 (ICAM-1). *Journal of Experimental Medicine*, **167**, 1323-1340.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A. & Springer, T.A. (1986) Induction by IL 1 and interferon-γ: Tissue distribution, Biochemistry, and function of a natural adherence molecule (ICAM-1). *The Journal of Immunology*, **137**, 245-254.

Dustin, M.L. & Springer, T.A. (1988) Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *The Journal of Cell Biology*, **107**, 321-331.

Dustin, M.L. & Springer, T.A. (1989) T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. *Nature*, **341**, 619-624.

Ebersole, J.L., Taubman, M.A., Smith, D.J. & Goodson, J.M. (1984) Gingival crevicular fluid antibody to oral microorganisms. I. Method of collection and analysis of antibody. *Journal of Periodontal Research*, **19**, 124-132.

Egelberg, J. (1966) Blood vessels of the dento-gingival junction. *Journal of Periodontal Research*, **1**, 163-179.

Egelberg, J. (1967) The topograph and permeability of vessels at the dento-gingival junction in dogs. *Journal of Periodontal Research*, **2**, supplement 1.

Elices, M.J., Osborn, L., Takada, Y., *et al.* (1990) VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell*, **60**, 577-584.

Ellegaard, B., Borregaard, N. & Ellegaard, J. (1984) Neutrophil chemotaxis and phagocytosis in juvenile periodontitis. *Journal of Periodontal Research*, **19**, 261-268.

Fantone, J.C. & Ward, P.A. (1982) Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. *American Journal of Pathology*, **197**, 395-418.

Farber, P.A. (1989) Chemotaxis of gingival neutrophils: Scanning electron microscopy. *Journal of Periodontology*, **60**, 540-543.

Finch, S.C. (1983) Neutrophil disorders: benign, quantitative abnormalities of neutrophils, ed. Williams, W.J. Bentler, E. Erslev, A.J. & Lichtman, M.A., *Haematology*, ed 3, pp 773-793. New-York: McGraw-Hill.

Fine, D.H. & Mandel, I.D. (1986) Indicators of periodontal disease activity: an evaluation. *Journal of Clinical Periodontology*, **13**, 533-546.

Fischman, S.L. (1986) Current status of indices of plaque. *Journal of Clinical Periodontology*, **13**, 371-374.

Fish, E.N. (1935) Parodontal disease: The pathology and treatment of chronic gingivitis. *British Dental Journal*, **58**, 531-554.

Fithian, E., Kung, P., Goldstein, G., Rubenfeld, M., Fenoglio, C. & Edelson, R. (1981) Reactivity of Langerhans cells with hybridoma antibody. *Proceedings of the National Academy of Sciences of the U. S. A.*, **78**, 2541-2544.

Freedman, H.L., Listgarten, M.A. & Taichman, N.S. (1968) Electron microscopic features of chronically inflamed human gingiva. *Journal of Periodontal Research*, **3**, 313-327.

Freemont, A.J. & Ford, W.L. (1985) Functional and morphological changes in post-capillary venules in relation to lymphocytic infiltration into BCG-induced granulomata in rat skin. *Journal of Pathology*, **147**, 1-12.

Fukuda, M., Spooncer, E., Oates, J.E., Dell, A. & Klock, J.C. (1984) Structure of sialylated fucosyl lactosaminoglycan isolated from human granulocytes. *The Journal of Biological Chemistry*, **259**, 10925-10935.

Gamble, J.R., Harlan, J.M., Klebanoff, S.J. & Vadas, M.A. (1985) Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. *Proceedings of the National Academy of Sciences of the U.S.A.*, **82**, 8667-8671.

Garant, P.R. (1976a) Light and electron microscopic observations of osteoclastic alveolar bone resorption in rats monoinfected with *Actinomyces Naeslundii*. *Journal of Periodontology*, **47**, 717-723.

Garant, P.R. (1976b) Plaque-neutrophil interaction in monoinfected rats as visualized by transmission electron microscopy. *Journal of Periodontology*, **47**, 132-138.

Garant, P.R. & Mulvihill, J.E. (1972) The fine structure of gingivitis in the beagle III. Plasma cell infiltration of the subepithelial connective tissue. *Journal of Periodontal Research*, **7**, 161-172.

Genco, R.J., Mashimo, P.A., Krygier, G. & Ellison, S.A. (1974) Antibody-mediated effects on the periodontium. *Journal of Periodontology*, **45**, 330-337.

Genco, R.J., Van Dyke, T.E., Levine, M.J., Nelson, R.D. & Wilson, M.E. (1986) Molecular factors influencing neutrophil defects in periodontal disease. *Journal of Dental Research*, **65**, 1379-1391.

Giannopoulou, C., Andersen, E., Demeurisse, C. & Cimasoni, G. (1992) Neutrophil elastase and its inhibitors in human gingival crevicular fluid during experimental gingivitis. *Journal of Dental Research*, **71**, 359-363.

Gillet, R., Cruchley, A. & Johnson, N.W. (1986) The nature of the inflammatory infiltrates in childhood gingivitis, juvenile periodontitis and adult periodontitis: Immunocytochemical studies using a monoclonal antibody to HLA Dr. *Journal of Clinical Periodontology*, **13**, 281-288.

Gillett, I.R., Johnson, N.W., Curtis, M.A., *et al.* (1990) The role of histopathology in the diagnosis and prognosis of periodontal diseases. *Journal of Clinical Periodontology*, **17**, 673-684.

Gilmore, S.J., Benson, E.M. & Kelly, J.W. (1991) T-cell subsets with a naive phenotype are selectively decreased in the peripheral blood of patients with mycosis Fungoides. *Journal of Investigative Dermatology*, **96**, 50-56.

Glossary of Periodontic Terms (1986) *Journal of Periodontology*, **57** (suppl), 1-31.

Goldhaber, P. & Giddon, D.B. (1964) Present concepts concerning the etiology and treatment of acute necrotizing ulcerative gingivitis. *The International Dental Journal*, **14**, 468-496.

Gonwa, T.A., Picker, L.J., Raff, H.V., Goyert, S.M., Silver, J. & Stobo, J.D. (1983) Antigen-presenting capabilities of human monocytes correlates with their expression of HLA-DS, an Ia determinant distinct from HLA-DR. *The Journal of Immunology*, **130**, 706-711.

Gowen, M., Wood, D.D., Ihrie, E.J., McGuire, M.K.B. & Russell, R.G.G. (1983) An interleukin 1 like factor stimulates bone resorption *in vitro*. *Nature*, **306**, 378-380.

Graber, N., Gopal, T.V., Wilson, D., Beall, L.D., Polte, T. & Newman, W. (1990) T cells bind to cytokine-activated endothelial cells via a novel, inducible sialoglycoprotien and endothelial leukocyte adhesion molecule-1. *The Journal of Immunology*, **145**, 819-830.

Grant, D.A. & Orban, B. (1960) Leukocytes in the epithelial attachment. *Journal of Periodontology*, **31**, 87-95.

Granger, G.A. & Williams, T.W. (1968) Lymphocyte cytotoxicity *in vitro*: Activation and release of a cytotoxic factor. *Nature*, **218**, 1253-1254.

Grbic, J. Celenti, R. Oshrain, R. & Lamster, I. (1991) The development of an ELISA for gamma-interferon in gingival crevicular fluid. *Journal of Dental Research*, **70**, pp591, abstract #2605.

Greenberg, M.S. (1984) Ulcerative vesicular and bullous lesions. In *Burket's Oral Medicine*, ed. Lynch, M.A., 8th ed, Ch. 5, pp 163-208. Philadelphia, PA: J.B. Lippincott Co.

Gregory, S.H. (1988) Endotoxin-stimulated mononuclear phagocytes secrete soluble factors with bone-resorbing activity. *Journal of Periodontal Research*, **23**, 7-12.

Griffiths, C.E.M. & Nickoloff, B.J. (1989) Keratinocyte intercellular adhesion molecule-1 (ICAM-1) expression precedes dermal T lymphocytic infiltration in Allergic Contact Dermatitis (*Rhus dermatitis*). *American Journal of Pathology*, **135**, 1045-1053.

Griffiths, G.S., Curtis, M.A. & Wilton, J.M.A. (1988) Selection of a filter paper with optimum properties for the collection of gingival crevicular fluid. *Journal of Periodontal Research*, **23**, 33-38.

Griffiths, C.E., Voorhees, J.J. & Nickoloff. B.J. (1989) Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: modulation by recombinant gamma interferon and tumor necrosis factor. *Journal of the American Academy of Dermatology*, **20**, 617-629.

Groves, R.W., Ross, E., Barker, J.N.W.N., Ross, J.S., Camp, R.D.R. & MacDonald, D.M. (1992) Effect of *in vivo* interleukin-1 on adhesion molecule expression in normal human skin. *Journal of Investigative Dermatology*, **98**, 384-387.

Haffajee, A.D., Socransky, S.S., Dzink, J.L., Taubman, M.A. & Ebersole, J.L. (1988) Clinical, microbiological and immunological features of subjects with refractory periodontal diseases. *Journal of Clinical Periodontology*, **15**, 390-398.

Hanazawa, S., Hirose, K., Ohmori, Y., Amano, S. & Kitano, S. (1988) *Bacteroides gingivalis* fimbriae stimulate production of Thymocyte-Activating Factor by human gingival fibroblasts. *Infection and Immunity*, **56**, 272-274.

Hanazawa, S., Nakada, K., Ohmori, Y., Miyoshi, T., Amano, S. & Kitano, S. (1985) Functional role of Interleukin 1 in periodontal disease: Induction of Interleukin 1 production by *Bacteroides gingivalis* lipopolysaccharide in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. *Infection and Immunity*, **50**, 262-270.

Harlan, J.M. (1985) Leukocyte-endothelial interactions. *Blood Journal of Haematology*, **65**, 513-525.

Harlan, J.M., Winn, R.K., Vedder, N.B., Doerschuk, C.M. & Rice, C.L. (1991) *In vivo* models of leukocyte adherence to endothelium. In *Adhesion: Its Role in Inflammatory Disease*, ed Harlan, J.M. & Liu, D.Y. Ch.6, pp 117-150. Oxford: W.H. Freeman & Co.

Harlan, J.M. & Liu, D.Y. (1991) *Adhesion: Its Role in Inflammatory Disease*. Oxford: W.H. Freeman & Co.

Harrist, T.J., Muhlbauer, J.E., Murphy, G.F., Mihm, M.C.Jr. & Bhan, A.K. (1983) T6 is superior to Ia (HLA-DR) as a marker for Langerhans cells and indeterminate cells in normal epidermis: A monoclonal antibody study. *Journal of Investigative Dermatology*, **80**, 100-103.

Haskard, D., Cavender, D., Beatty, P., Springer, T. & Ziff, M. (1986) T lymphocyte adhesion to endothelial cells: Mechanisms demonstrated by anti-LFA-1 monoclonal antibodies. *The Journal of Immunology*, **137**, 2901-2906.

Haskard, D.O., Cavender, D., Fleck, R.M., Sontheimer, R. & Ziff, M. (1987) Human dermal microvascular endothelial cells behave like umbilical vein endothelial cells in T-cell adhesion studies. *Journal of Investigative Dermatology*, **88**, 340-344.

Hay, J.B., Lachmann, P.J. & Trnka, Z. (1973) The appearance of migration inhibition factor and a mitogen in lymph draining tuberculin reactions. *European Journal of Immunology*, **3**, 127-131.

Hellden, L. & Lindhe, J. (1973) Enhanced emigration of crevicular leukocytes mediated by factors in human dental plaque. *Scandinavian Journal of Dental Research*, **81**, 123-129.

Helle, M., Boeije, L. & Aarden, L.A. (1988) Functional discrimination between Interleukin 6 and Interleukin 1. *European Journal of Immunology*, **18**, 1535-1540.

Hemler, M.E., Glass, D., Coblyn, J.S. & Jacobson, J.S. (1986) Very late activation antigens on rheumatoid synovial fluid T lymphocytes: Association with stages of T cell activation. *Journal of Clinical Investigations*, **78**, 696-702.

Herman, P.G., Yamamoto, I. & Mellins, H.Z. (1972) Blood microcirculation in the lymph node during the primary immune response. *Journal of Experimental Medicine*, **136**, 697-714.

Hetherington, S.V., Spitznagel, J.K. & Quie, P.G. (1983) An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin. *Journal of Immunological Methods*, **65**, 183-190.

Hildreth, J.E.K., Gotch, F.M., Hildreth, P.D.K. & McMichael, A.J. (1983) A human lymphocyte-associated antigen involved in cell-mediated lympholysis. *European Journal of Immunology*, **13**, 202-208.

Hitzig, C., Monteil, R.A., Charbit, Y. & Teboul, M. (1989) Quantification of T6+ and HLA/DR+ Langerhans cells in normal and inflammed human gingiva. *Journal de Biologie Buccale*, **17**, 103-108.

Hoffman, R.A., Kung, P.C., Hansen, W.P. & Goldstein, G. (1980) Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. *Proceedings of the National Academy of Sciences of the U.S.A.*, **77**, 4914-4917.

Hönig, J., Rordorf-Adam, C., Siegmund, C., Wiedemann, W. & Erard, F. (1989) Interleukin-1 beta (IL-1β) concentration in gingival tissue from periodontitis patients and healthy control subjects. *Journal of Periodontal Research*, **24**, 362-367.

Hopkins, S.J. & Humphreys, M. (1989) Simple, sensitive and specific bioassay for Interleukin 1. *Journal of Immunological Methods*, **120**, 271-276.

Horton, J.E., Oppenheim, J.J. & Mergenhagen, S.E. (1974) A role for cell-mediated immunity in the pathogenesis of periodontal disease. *Journal of Periodontology*, **45**, 351-360.

Hsu, S.-M., Raine, L. & Fanger, H. (1981) A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. *American Journal of Clinical Periodontology*, **75**, 734-738.

Hynes, R.O. (1987) Integrins: A family of cell surface receptors. *Cell*, **48**, 549-555.

Inaba, K. & Steinman, R.M. (1984) Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. *Journal of Experimental Medicine*, **160**, 1717-1735.

Ishii, T., Walsh, L.J., Seymour, G.J. & Powell, R.N. (1990) Modulation of Langerhans cell surface antigen expression by recombinant cytokines. *Journal of Oral Pathology and Medicine*, **19**, 355-359.

Isaacs, A. & Lindenmann, J. (1987) Virus interference. I. The interferon. BY A.Isaacs and J. Lindenmann, 1957 [classical article]. *Journal of Interferon Research*, **7**, 429-438.

Ivanyi, L. & Lehner, T. (1970) Stimulation of lymphocyte transformation by bacterial antigens in patients with periodontal disease. *Archives of Oral Biology*, **15**, 1089-1096.

Jalkanen, S., Wu, N., Bargatze, R.F. & Butcher, E.C. (1987) Human lymphocyte and lymphoma homing receptors. *Annual Review of Medicine*, **38**, 467-476.

James, W.W. & Counsell, A. (1927) A histological investigation into "so called Pyorrhoea Alveolaris". *British Dental Journal*, **48**, 1237-1252.

Jandinski, J.J. (1988) Osteoclast activating factor is now interleukin-1 beta: Historical perspective and biological implications. *Journal of Oral Pathology*, **17**, 145-152.

Jandinski, J.J., Stashenko, P., Feder, L.S., *et al.* (1991) Localization of interleukin-1β in human periodontal tissue. *Journal of Periodontology*, **62**, 36-43.

Jenkins, W.M.M. & Kinane, D.F. (1989) The 'high risk' group in periodontitis. *British Dental Journal*, **167**,168-171.

Joachim, F., Barber, P., Newman, H.N. & Osborn, J. (1990) The plasma cell at the advancing front of the lesion in chronic periodontitis. *Journal of Periodontal Research*, **25**, 49-59.

Johannessen, A.C., Nilsen, R., Knudsen, G.E. & Kristoffersen, T. (1986) *In situ* characterization of mononuclear cells in human chronic marginal periodontitis using monoclonal antibodies. *Journal of Periodontal Research*, **21**, 113-127.

Jutila, M.A., Berg, E.L., Kishimoto, T.K., *et al.* (1989) Inflammation-induced endothelial cell adhesion to lymphocytes, neutrophils and monocytes. Role of homing receptors and other adhesion molecules. *Transplantation*, **48**, 727-731.

Kabashima, H., Maeda, K., Iwamoto, Y., *et al.* (1990) Partial characterization of an interleukin-1-like factor in human gingival crevicular fluid from patients with chronic inflammatory periodontal disease. *Infection and Immunity*, **58**, 2621-2627.

Kahnberg, K.-E. & Hellden, L. (1977) Effect of topically applied neutrophil leukocyte extract on normal gingiva of dogs. *Scandinavian Journal of Dental Research*, **85**, 155-163.

Kahnberg, K.-E., Lindhe, J. & Hellden, L. (1976) Initial gingivitis induced by topical application of plaque extracts. A histometric study in dogs with normal gingivae. *Journal of Periodontal Research*, **11**, 218-225.

Kalkwarf, K.L. (1978) Effect of oral contraceptive therapy on gingival inflammation in humans. *Journal of Periodontology*, **49**, 560-563.

Karring, T. & Löe, H. (1967) The blood supply to the periodontium. *Journal of Periodontal Research*, **2**, 74-75.

Katsuragi, Y., Matsuda, N., Nakamura, M. & Murayama, Y. (1988) Neutrophil functions in patients with severe periodontal disease. *Advances in Dental Research*, **2**, 359-363.

Katz, J., Goultschin, J., Benoliel, R. & Schlesinger, M. (1988) T lymphocyte subsets in rapidly progressive periodontitis. *Journal of Clinical Periodontology*, **15**, 266-268.

Katz, S.I., Tamaki, K. & Sachs D.H. (1979) Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature*, **282**, 324-326.

Kelly, R.H., Wolstencroft, R.A., Dumonde, D.C. & Balfour, B.M. (1972) Role of lymphocyte activation products (LAP) in cell-mediated immunity: II. Effects of lymphocyte activation products on lymph node architecture and evidence for peripheral release of LAP following antigenic stimulation. *Clinical and Experimental Immunology*, **10**, 49-65.

Kinane, D.F., Cullen, C.F., Johnston, F.A. & Evans, C.W. (1989a) Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (I). Leading front analysis in Boyden chambers. *Journal of Clinical Periodontology*, **16**, 242-246.

Kinane, D.F., Cullen, C.F., Johnston, F.A. & Evans, C.W. (1989b) Neutrophil chemotactic behaviour in patients with early-onset forms of periodontitis (II). Assessment using the under agarose technique. *Journal of Clinical Periodontology*, **16**, 247-251.

Kinane, D.F., Johnston, F.A. & Evans, C.W. (1989) Depressed helper-to-suppressor T-cell ratios in early-onset forms of periodontal disease. *Journal of Periodontal Research*, **24**, 161-164.

Kirnbauer, R., Charvat, B., Schauer, E., *et al.* (1992) Modulation of intercellular adhesion molecule-1 expression on human melanocytes and melanoma cells: Evidence for a regulatory role of IL-6, Il-7, TNFβ, and UVB light. *Journal of Investigative Dermatology*, **98**, 320-326.

Klareskog, L., Tjernlund, U.M., Forsum, U. & Peterson, P.A. (1977) Epidermal Langerhans cells express Ia antigens. *Nature*, **268**, 248-250.

Knight, G.M. & Wade, A.B. (1974) The effects of hormonal contraceptives on the human periodontium. *Journal of Periodontal Research*, **9**, 18-22.

Koch, A.E., Burrows, J.C., Haines, G.K., Carlos, T.M., Harlan, J.M. & Leibovich , S.J. (1991) Immunolocalization of endothelial and leukocyte adhesion molecules in human rheumatoid and osteoarthritic synovial tissues. *Laboratory Investigations*, **64**, 313-320.

Kornman, K.S. (1987) Nature of periodontal diseases: Assessment and diagnosis. *Journal of Periodontal Research*, **22**, 192-204.

Kowashi, Y., Jaccard, F. & Cimasoni, G. (1980) Sulcular polymorphonuclear leukocytes and gingival exudate during experimental gingivitis in man. *Journal of Periodontal Research*, **15**, 152-158.

Kowolik, M.J. & Raeburn, J.A. (1980) Functional integrity of gingival crevicular neutrophil polymorphonuclear leucocytes as demonstrated by nitroblue tetrazolium reduction. *Journal of Periodontal Research*, **15**, 483-491.

Krutmann, J., Czech, W., Parlow, F., *et al.* (1992) Ultraviolet radiation effects on human keratinocyte ICAM-1 expression: UV-induced inhibition of cytokine-induced ICAM-1 mRNA expression is transient, differentially restored for IFNγ versus TNFα, and followed by ICAM-1 induction via a TNFα-like pathway. *Journal of Investigative Dermatology*, **98**, 923-928.

Krutmann, J., Köck, A., Schauer, E., *et al.* (1990) Tumor necrosis factor β and ultraviolet radiation are potent regulators of human keratinocyte ICAM-1 expression. *Journal of Investigative Dermatology*, **95**, 127-131.

Kupper, T.S., Ballard, D.W., Chua, A.O., *et al.* (1986) Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1α mRNA and β mRNA. Keratinocyte epidermal cell-derived thymocyte-activity factor is identical to interleukin 1. *Journal of Experimental Medicine*, **164**, 2095-2100.

Kyle, R.A. & Linman, J.W. (1970) Gingivitis and chronic idiopathic neutropenia: report of two cases, *Mayo Clinic Proceedings*, **45**, 494-504.

Lally, E.T., Baehni, P.C. & McArthur, W.P. (1980) Local immunoglobulin synthesis in periodontal disease. *Journal of Periodontal Research*, **15**, 159-164.

Lampert, F. & Fesseler, A. (1975) Periodontal changes during chronic benign granulocytopenia in childhood. A case report. *Journal of Clinical Periodontology*, **2**, 105-110.

Lampson, L.A. & Levy, R. (1980) Two populations of Ia-like molecules on a human B cell line. *The Journal of Immunology*, **125**, 293-299.

Lang, N.P. & Smith, F.N. (1977) Lymphocyte blastogenesis to plaque antigens in human periodontal disease. I. Populations of varying severity of disease. *Journal of Periodontal Research*, **12**, 298-309.

Lareau, D.E., Herzberg, M.C. & Nelson, R.D. (1984) Human neutrophil migration under agarose to bacteria associated with the development of gingivitis. *Journal of Periodontology*, **55**, 540-549.

Larson, R.S. & Springer, T.A. (1990) Structure and function of leukocyte integrins. *Immunological Reviews*, **114**, 181-217.

Lavine, W.S., Maderazo, E.G., Stolman, J., *et al.* (1979) Impaired neutrophil chemotaxis in patients with juvenile and rapidly progressing periodontitis. *Journal of Periodontal Research*, **14**, 10-19.

Le, J. & Vilcek, J. (1987) Tumour necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. *Laboratory Investigation*, **56**, 234-248.

Ledbetter, J.A., Rose, L.M., Spooner, C.E., Beatty, P.G., Martin, P.J. & Clark, E.A. (1985) Antibodies to common leukocyte antigen p220 influence human T cell proliferation by modifying IL 2 receptor expression. *The Journal of Immunology*, **135**, 1819-1833.

Leeuwenberg, J.F.M., Von Asmuth, E.J.U., Jeunhomme, T.M.A.A. & Buurman, W.A. (1990) IFN-γ regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells *in vitro*. *The Journal of Immunology*, **145**, 2110-2114.

Lehner, T. (1972) Cell-mediated immune responses in oral disease: A review. *Journal of Oral Pathology*, **1**, 39-58.

Levine, S. (1959) Chronic familial neutropenia with marked periodontal lesions. *Oral Surgery, Oral Medicine and Oral Pathology*, **12**, 310-314.

Lewis, R.E., Buchsbaum, M., Whitaker, D. & Murphy, G.F. (1989) Intercellular adhesion molecule expression in the evolving human cutaneous delayed hypersensitivity reaction. *Journal of Investigative Dermatology*, **93**, 672-677.

Lindhe, J., Hamp, S-E. & Löe, H. (1973) Experimental periodontitis in the Beagle dog. *Journal of Periodontal Research*, **8**, 1-10.

Lindhe, J., Hamp, S-E. & Löe, H. (1975) Plaque induced periodontal disease in beagle dogs. A 4-year clinical roentgenographical and histometrical study. *Journal of Periodontal Research*, **10**, 243-255.

Lindhe, J. & Rylander, H. (1975) Experimental gingivitis in young dogs. *Scandinavian Journal of Dental Research*, **83**, 314-326.

Lindhe, J., Schroeder, H.E., Page, R.C., Münzel-Pedrazzoli, S. & Hugoson, A. (1974) Clinical and stereologic analysis of the course of early gingivitis in dogs. *Journal of Periodontal Research*, **9**, 314-330.

Lipsky, P.E. & Rosenthal A.S. (1975) Macrophage-lymphocyte interaction. II Antigen-mediated physical interactions between immune guinea pig lymph node lymphocytes and syngeneic macrophages. *Journal of Experimental Medicine*, **141**, 138-154.

Listgarten, M.A. (1986) Pathogenesis of periodontitis. *Journal of Clinical Periodontology*, **13**, 418-425.

Listgarten, M.A. (1988) The role of dental plaque in gingivitis and periodontitis. *Journal of Clinical Periodontology*, **15**, 485-487.

Listgarten, M.A. & Ellegaard, B. (1973) Experimental gingivitis in the monkey. Relationship of leucocyte in junctional epithelium, sulcus depth and connective tissue inflammation scores. *Journal of Periodontal Research*, **8**, 199-214.

Listgarten, M.A. & Heneghan, J.B. (1971) Chronic inflammation in the gingival tissue of germ-free dogs. *Archives of Oral Biology*, **16**, 1207-1213.

Listgarten, M.A. & Heneghan, J.B. (1973) Observations on the periodontium and acquired pellicle of adult germfree dogs. *Journal of Periodontology*, **44**, 85-91.

Lo, S.K., Van Seventer, G.A., Levin, S.M. & Wright, S.D. (1989) Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. *The Journal of Immunology*, **143**, 3325-3329.

Lobene, R.R., Weatherford, T., Ross, N.M., Lamm, R.A. & Menaker, L. (1986) A modified gingival index for use in clinical trials. *Clinical Preventive Dentistry*, **8**, 3-6.

Löe, H. (1965) Periodontal changes in pregnancy. *Journal of Periodontology*, **36**, 209-217.

Löe, H., Anerud, A., Boysen, H. & Morrison, E. (1986) Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *Journal of Clinical Periodontology*, **13**, 431-440.

Löe, H., Anerud, A., Boysen, H. & Smith, M. (1978) The natural history of periodontal disease in man. The rate of periodontal destruction before 40 years of age. *Journal of Periodontology*, **49**, 607-620.

Löe, H. & Holm-Pedersen, P. (1965) Absence and presence of fluid from normal and inflamed gingivae. *Periodontics*, **3**, 171-177.

Löe, H. & Karring, T. (1971). The three-dimensional morphology of the epithelium-connective tissue interface of the gingiva as related to age and sex. *Scandinavian Journal of Dental Research*, **79**, 315-326.

Löe, H. & Silness, J. (1963) Periodontal disease in pregnancy I. Prevalence and severity. *Acta Odontologica Scandinavica*, **21**, 533-551.

Löe, H., Theilade, E. & Jensen, S.B. (1965) Experimental gingivitis in man. *Journal of Periodontology*, **36**, 177-187.

Longhurst, P., Gillett, R. & Johnson, N.W. (1980) Electron microscope quantitation of inflammatory infiltrates in childhood gingivitis. *Journal of Periodontal Research*, **15**, 255-266.

Lovdal, A., Arno, A. & Waerhaug, J. (1958) Incidence of clinical manifestations of periodontal disease in light of oral hygiene and calculus formation. *Journal of the American Dental Association*, **56**, 21-33.

Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L. & Marks, R.M. (1990) ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. *Cell*, **63**, 475-484.

Lundström, A., Johansson, L.-A. & Hamp, S-E. (1984) Effect of combined systemic antimicrobial therapy and mechanical plaque control in patients with recurrent periodontal disease. *Journal of Clinical Periodontology*, **11**, 321-330.

Lyons, H., Bernier, H. & Goldman, H.M. (1959) Committee Reports....Nomenclature and classification committee. *Journal of Periodontology*, **30**, 74-77.

Mackay, C.R. (1991) Skin-seeking memory T cells. *Nature*, **349**, 737-738.

Mackler, B.F., Frostad, K.B., Robertson, P.B. & Levy, B.M. (1977) Immunoglobulin bearing lymphocyte and plasma cells in human periodontal disease. *Journal of Periodontal Research*, **12**, 37-45.

Majewski, S., Hunzelmann, N., Johnson, J.P., *et al.* (1991) Expression of intercellular adhesion molecule-1 (ICAM-1) in the skin of patients with Systemic Scleroderma. *Journal of Investigative Dermatology*, **97**, 667-671.

Maiden, M.F., Carman, R.J., Curtis, M.A., *et al.* (1990) Detection of high-risk groups and individuals for periodontal diseases: laboratory markers based on the microbiological analysis of subgingival plaque. *Journal of Clinical Periodontology*, **17**, 1-13.

Malberg, K., Mölle, A., Streuer, D. & Gängler, P. (1992) Determination of lymphocyte populations and subpopulations extracted from chronically inflamed human periodontal tissues. *Journal of Clinical Periodontology*, **19**, 155-158.

Mann, M.V. (1963) The correlation of gingivitis, pocket depth and exudate from the gingival crevice. *Journal of Periodontology*, **34**, 379-387.

Manson, J.D. & Lehner, T. (1974) Clinical features of juvenile periodontitis (periodontosis). *Journal of Periodontology*, **45**, 636-640.

March, C.J., Mosley, B., Larsen, A., *et al.* (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs, *Nature*, **315**, 641-647.

Marshall-Day, C.D., Stephens, R.G. & Quigley, L.F.Jr. (1955) Periodontal disease: Prevalence and incidence. *Journal of Periodontology*, **26**, 185-203.

Marucha, P.T., Zeff, R.A. & Kreutzer, D.L. (1991) Regulation of IL-1β gene expression in human peripheral blood PMN. *Journal of Periodontal Research*, **26**, 264-267.

Masada, M.P., Persson, R., Kenney, J.S., Lee, S.W., Page, R.C. & Allison, A.C. (1990) Measurement of interleukin-1α and -1β in gingival crevicular fluid: Implications for the pathogenesis of periodontal disease. *Journal of Periodontal Research*, **25**, 156-163.

Masinovsky, B., Urdal, D. & Gallatin, W.M. (1990) IL-4 acts synergistically with IL-1β to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *The Journal of Immunology*, **145**, 2886-2895.

Matthews, J.N.S., Altman, D.G., Campbell, M.J. & Royston, P. (1990) Analysis of serial measurements in medical research. *British Medical Journal*, **300**, 230-235.

McCarthy, P.L. & Shklar, G. (1980) *Diseases of the Oral Mucosa*, 2nd ed. Philadelphia, PA: Lea & Ferbiger.

McFall, W.T.Jr. (1982) Tooth loss in 100 treated patients with periodontal disease. A long-term study. *Journal of Periodontology*, **53**, 539-549.

Meikle, M.C., Atkinson, S.J., Ward, R.V., Murphy, G. & Reynolds, J.J. (1989) Gingival fibroblasts degrade type 1 collagen films when stimulated with tumor necrosis factor and interleukin 1: Evidence that breakdown is mediated by metalloproteinases. *Journal of Periodontal Research*, **24**, 207-213.

Meikle, M.C., Heath, J.K. & Reynolds J.J. (1986) Advances in understanding cell interactions in tissue resorption. Relevances to the pathogenesis of periodontal diseases and a new hypothesis. *Journal of Oral Pathology*, **15**, 239-250.

Meng, H.X. & Zheng, L.F. (1989) T cells and T-cell subsets in periodontal diseases. *Journal of Periodontal Research*, **24**, 121-126.

Mergenhagen, S.E. (1984) Thymocyte activating factor(s) in human gingival fluids. *Journal of Dental Research*, **63**, 461-464.

Messadi, D.V., Pober, J.S., Fiers, W., Gimbrone, M.A.Jr. & Murphy, G.F. (1987) Induction of an activation antigen on postcapillary venular endothelium in human skin organ culture. *The Journal of Immunology*, **139**, 1557-1562.

Miyasaki, K.T. (1991) The neutrophil: Mechanisms of controlling periodontal bacteria. *Journal of Periodontology*, **62**, 761-774.

Mochan, E., Armor, L. & Sporer, R. (1988) Interleukin 1 stimulation of plasminogen activator production in cultured gingival fibroblasts. *Journal of Periodontal Research*, **23**, 28-32.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., *et al.* (1982) Bacteriology of experimental gingivitis in young adult humans. *Infection and Immunity*, **38**, 651-667.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., *et al.* (1984) Bacteriology of experimental gingivitis in children. *Infection and Immunity*, **46**, 1-6.

Morimoto, C., Hafler, D.A., Weiner, H.L., *et al.* (1987) Selective loss of the suppressor-inducer T-cell subset in progressive multiple sclerosis. Analysis with anti-2H4 monoclonal antibody. *The New England Journal of Medicine*, **316**, 67-72.

Morimoto, C., Letvin, N.L., Distaso, J.A., Aldrich, W.R. & Schlossman, S.F. (1985) The isolation and characterization of the human suppressor inducer T cell subset. *The Journal of Immunology*, **134**, 1508-1515.

Moser, R., Schleiffenbaum, B., Groscurth, P. & Fehr, J. (1989) Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. *Journal of Clinical Investigations*, **83**, 444-455.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**, 55-63.

Moulon, C., Peguet-Navarro, J. & Schmitt, D. (1991) A potential role for CD1a molecules on human epidermal Langerhans cells in allogeneic T-cell activation. *Journal of Investigative Dermatology*, **97**, 524-528.

Mouton, C., Hammond, P.G., Slots, J. & Genco, R.J. (1981) Serum antibodies to oral *Bacteriodes asaccharolyticus (Bacteriodies gingivalis)*: Relationship to age and periodontal disease. *Infection and Immunity*, **31**, 182-192.

Mulvihill, J.E., Susi, F.R., Shaw, J.H. & Goldhgreenbaber, P. (1967) Histological studies of the periodontal syndrome in rice rats and the effects of penicillin. *Archives of Oral Biology*, **12**, 733-744.

Munro, J.M., Pober, J.S. & Cotran, R.S. (1989) Tumour necrosis factor and interferon-γ induce distinct patterns of endothelial cell activation and associated leukocyte accumulation in the skin of *Papio anubis*. *American Journal of Pathology*, **135**, 121-133.

Munro, J.M., Pober, J.S. & Cotran, R.S. (1991) Recruitment of neutrophils in the local endotoxin response: Association with *de novo* endothelial expression of endothelial leukocyte adhesion molecule-1. *Laboratory Investigations*, **64**, 295-299.

Murphy, G.F., Bhan, A.K., Sato, S., Mihm, M.C. & Harrist, T.J. (1981) A new immunologic marker for human Langerhans cells. *The New England Journal of Medicine*, **304**, 791-792.

Ness, K.H., Morton, T.H. & Dale, B.A. (1987) Identification of Merkel cells in oral epithelium using antikeratin and antineuroendocrine monoclonal antibodies. *Journal of Dental Research*, **66**, 1154-1158.

Newcomb, G.M. & Powell R.N. (1986a) The ultrastructure of human gingival Langerhans cells in health and disease. *Archives of Oral Biology*, **31**, 727-734.

Newcomb, G.M. & Powell, R.N. (1986b) Human gingival Langerhans cells in health and disease. *Journal of Periodontal Research*, **21**, 640-652.

Newcomb, G.M. & Powell, R.N. (1988) Human gingival Langerhans cells as accessory cells in mitogen induced T cell responses. *Journal of Periodontology*, **59**, 811-815.

Newcomb, G.M., Seymour, G.J. & Powell, R.N. (1982) Association between plaque accumulation and Langerhans cell numbers in the oral epithelium of attached gingiva. *Journal of Clinical Periodontology*, **9**, 297-304.

Newman, H.N. (1990) Plaque and chronic inflammatory periodontal disease. A question of ecology. *Journal of Clinical Periodontology*, **17**, 533-541.

Nightingale, G. & Hurley, J.V. (1978) Relationship between lymphocyte emigration and vascular endothelium in chronic inflammation. *Pathology*, **10**, 27-44.

Norris, P., Poston, R.N., Thomas, D.S., Thornhill, M., Hawk, J. & Haskard, D.O. (1991) The expression of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: A comparison of ultraviolet B erythema and delayed hypersensitivity. *Journal of Investigative Dermatology*, **96**, 763-770.

Norton, A.J., Ramsay, A.D., Smith, S.H. & Beverley, P.C.L. (1986) Monoclonal antibody (UCHL1) that recognises normal and neoplastic T cells in routinely fixed tissues. *Journal of Clinical Pathology*, **39**, 399-405.

O'Neill, P.A. & Woodson, D.L. (1986) Lymphokine production by human gingival lymphocytes. *Journal of Periodontal Research*, **21**, 338-350.

O'Neill, P.A., Woodson, D.L. & Mackler, B.F. (1982) Functional characterization of human gingival lymphocytes Cytotoxic activity. *Journal of Periodontal Research*, **17**, 50-59.

Offenbacher, S., Odle, B.M., Gray, R.C. & Van Dyke, T.E. (1984) Crevicular fluid prostaglandin E levels as a measure of the periodontal disease status of adult and juvenile periodontitis patients. *Journal of Periodontal Research*, **19**, 1-13.

Ogawa, T., Tarkowski, A., McGhee, M.L., *et al.* (1989) Analysis of human IgG and IgA subclass antibody-secreting cells from localised chronic inflammatory tissue. *The Journal of Immunology*, **142**, 1150-1158.

Ohta, H., Kato, K., Fukui, K. & Gottschal, J.C. (1991) Microbial interactions and the development of periodontal disease. *Journal Of Periodontal Research*, **26**, 255-257.

Okada, H., Kassai, Y. & Kida, T. (1984) T lymphocyte subsets in the inflamed gingiva of human adult periodontitis. *Journal of Periodontal Research*, **19**, 595-598.

Okada, H., Kida, T. & Yamagami, H. (1983) Identification and distribution of immunocompetent cells in inflamed gingiva of human chronic periodontitis. *Infection and Immunity*, **41**, 365-374.

Okada, H. & Silverman, M.S. (1979) Chemotactic activity in periodontal disease I. The role of complement in monocyte chemotaxis. *Journal of Periodontal Research*, **14**, 20-25.

Oliver, R.C., Holm-Pedersen, P. & Löe, H. (1969) The correlation between clinical scoring, exudate measurements and microscopic evaluation of inflammation of the gingiva. *Journal of Periodontology*, **40**, 201-209.

Oppenheimer-Marks, N., Davis, L.S. & Lipsky, P.E. (1990) Human T lymphocyte adhesion to endothelial cells and transendothelial migration. Alteration of receptor use relates to the activation status of both the T cell and the endothelial cell. *The Journal of Immunology*, **145**, 140-148.

Oppenheimer-Marks, N. & Ziff, M. (1988) Migration of lymphocytes through endothelial cell monolayers: Augmentation by interferon-γ. *Cellular Immunology*, **114**, 307-323.

Orban, B. (1948) Clinical and histologic study of the surface characteristics of the gingiva. *Oral Surgery, Oral Medicine and Oral Pathology*, **1**, 827-841.

Osborn, L. (1990) Leukocyte adhesion to endothelium in inflammation. *Cell*, **62**, 3-6.

Osborn, L., Hession, C., Tizard, R., *et al.* (1989) Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell*, **59**, 1203-1211.

Page, R.C. (1986) Gingivitis. *Journal of Clinical Periodontology*, **13**, 345-355.

Page, R.C., Ammons, W.F., Schectman, L.R. & Dillingham, L.A. (1974) Collagen fibre bundles of the normal marginal gingiva in the marmoset. *Archives of Oral Biology*, **19**, 1039-1043.

Page, R.C., Beatty, P. & Waldrop, T.C. (1987) Molecular basis for the functional abnormality in neutrophils from patients with generalized prepubertal periodontitis. *Journal of Periodontal Research*, **22**, 182-183.

Page, R.C., Bowen, T., Altman, L., *et al.* (1983) Prepubertal periodontitis. I. Definition of a clinical disease entity. *Journal of Periodontology*, **54**, 257-271.

Page, R.C. & Schroeder, H.E. (1973) Biochemical aspects of the connective tissue alterations in inflammatory gingival and periodontal disease. *The International Dental Journal*, **23**, 455-469.

Page, R.C. & Schroeder, H.E. (1976) Pathogenesis of inflammatory periodontal disease. A summary of current work. *Laboratory Investigations*, **34**, 235-249.

Page, R.C. & Schroeder, H.E. (1981) Current status of the host response in chronic marginal periodontitis. *Journal of Periodontology*, **52**, 477-491.

Page, H. & Schroeder, H.E. (1982) *Periodontitis in Man and other Animals. A Comparative Review.* Basel, Switzerland: S. Karger.

Page, R.C., Simpson, D.M. & Ammons, W.F. (1975) Host tissue response in chronic inflammatory periodontal disease. IV The periodontal and dental status of a group of aged great apes. *Journal of Periodontology*, **46**, 144-155.

Page, R.C., Sims, T.J., Geissler, F., Altman, L.C. & Baab, D.A. (1984) Abnormal leukocyte motility in patients with early-onset periodontitis. *Journal of Periodontal Research*, **19**, 591-594.

Page, R.C., Sims, T.J., Geissler, F., Altman, L.C. & Baab, D.A. (1985) Defective neutrophil and monocyte motility in patients with early onset periodontitis. *Infection and Immunity*, **47**, 169-175.

Parrott, D.M.V. & Wilkinson, P.C. (1981) Lymphocyte locomotion and migration. *Progress in Allergy*, **28**, 193-284.

Payne, W.A., Page, R.C., Ogilvie, A.L. & Hall, W.B. (1975) Histopathologic features of the initial and early stages of experimental gingivitis in man. *Journal of Periodontal Research*, **10**, 51-64.

Perala, D.G., Chapman, R.J., Gelfand, J.A., Callahan, M.V., Adams, D.F. & Lie, T. (1992) Relative production of IL-1β and TNFα by mononuclear cells after exposure to dental implants. *Journal of Periodontology*, **63**, 426-430.

Perry, N.A., Deffner, N.F. & Sheridan, P.J. (1973) Atypical gingivostomatitis. *Archives of Dermatology*, **107**, 872-978.

Phillips, M.L., Nudelman, E., Gaeta, F.C.A., *et al.* (1990) ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, Sialyl-Le^x. *Science*, **250**, 1130-1132.

Picker, L.J., Kishimoto, T.K., Smith, C.W., Warnock, R.A. & Butcher, E.C. (1991) ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature*, **349**, 796-799.

Pitzalis, C., Kingsley, G., Haskard, D. & Panayi, G. (1988) The preferential accumulation of helper-inducer T lymphocytes in inflammatory lesions: evidence for regulation by selective endothelial and homotypic adhesion. *European Journal of Immunology*, **18**, 1397-1404.

Pitzalis, C., Kingsley, G., Murphy, J. & Panayi, G. (1987) Abnormal distribution of the helper-inducer and suppressor-inducer T-lymphocyte subsets in the rheumatoid joint. *Clinical Immunology and Immunopathology*, **45**, 252-258.

Platanias, L.C. & Vogelzang, N.J. (1990) Interleukin-1: biology, pathophysiology, and clinical prospects. *American Journal of Medicine*, **89**, 621-629.

Pober, J.S. (1988) Cytokine-mediated activation of vascular endothelium. Physiology and pathology. *American Journal of Pathology*, **133**, 426-433.

Pober, J.S., Bevilacqua, M.P., Mendrick, D.L., Lapierre, L.A., Fiers, W. & Gimbrone, M.A.Jr. (1986) Two distinct monokines, Interleukin 1 and Tumor Necrosis Factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *The Journal of Immunology*, **136**, 1680-1687.

Pober, J.S., Gimbrone, M.A.Jr., Collins, T., *et al.* (1984) Interactions of T lymphocytes with human vascular endothelial cells: Role of endothelial cell surface antigens. *Immunobiology*, **168**, 483-494.

Porcelli, S., Brenner, M.B., Greenstein, J.L., Balk, S.P., Torhost, C. & Bleicher, P.A. (1989) Recognition of cluster of differentiation 1 antigens by human CD4-CD8-cytolytic T lymphocytes. *Nature*, **341**, 447-449.

Postlethwaite, A.E., Lachman, L.B., Mainardi, C.L. & Kang, A.H. (1983) Interleukin-1 stimulation of collagenase production by cultured fibroblasts. *Journal of Experimental Medicine*, **157**, 801-806.

Poulter, L.W., Duke, O., Panayi, G.S., Hobbs, S., Raffery, M.J. & Janossy, G. (1985) Activated T lymphocytes of the synovial membrane in rheumatoid arthritis and other arthropathies. *Scandinavian Journal of Immunology*, **22**, 683-690.

Pulford, K.A., Erber, W.N., Crick, J.A. *et al.* (1988) Use of monoclonal antibody against human neutrophil elastase in normal and leukaemic myeloid cells. *Journal of Clinical Pathology*, **41**, 853-860.

Raisz, L.G., Sandberg, A.L., Goodson, J.M., Simmons, H.A. & Mergenhagen, S.E. (1974) Complement-dependent stimulation of prostaglandin synthesis and bone resorption. *Science*, **185**, 789-791.

Ramfjord, S.P. & Arbor, A. (1959) Indices for prevalence and incidence of periodontal disease. *Journal of Periodontology*, **30**, 51-59.

Ranney, R.R., Ruddy, S., Tew, J.G., Welshimer, H.J., Palcanis, K.G. & Segreti, A. (1981) Immunological studies of young adults with severe periodontitis. I. Medical evaluation and humoral factors. *Journal of Periodontal Research*, **16**, 390-402.

Ranney, R.R., Yanni, N.R., Burmeister, J.A. & Tew, J.G. (1982) Relationship between attachment loss and precipitating serum antibody to *Actinobacillus actinomycetemcomitans* in adolescents and young adults having severe periodontal destruction. *Journal of Periodontology*, **53**, 1-7.

Reed, M.J., Neiders, M.E. & Genco, R.J. (1976) Synergistic effects on DNA synthesis in peripheral blood lymphocytes of mitogens and dental plaque sonicates in man and macaque monkeys. *Archives of Oral Biology*, **21**, 441-445.

Reinhardt, R.A., Bolton, R.W., McDonald, T.L., Bubois, L.M. & Kaldahl, W.B. (1988) *In situ* lymphocyte subpopulations from active verses stable periodontal sites. *Journal of Periodontology*, **59**, 656-670.

Rice, G.E. & Bevilacqua, M.P. (1989) An inducible endothelial cell surface glycoprotein mediates melanoma adhesion. *Science*, **246**, 1303-1306.

Rice, G.E., Munro, J.M. & Bevilacqua, M.P. (1990) Inducible cell adhesion molecule 110 (INCAM-110) is an endothelial receptor for lymphocytes. A CD11/CD18-independent adhesion mechanism. *Journal of Experimental Medicine*, **171**, 1369-1374.

Rice, W.G., Ganz, T., Kinkade, J.M.Jr., Selsted, M.E., Lehrer, R.I. & Parmley, R.T. (1987) Defensin-rich dense granules of human neutrophils. *Blood Journal of Haematology*, **70**, 757-765.

Richards, D. & Rutherford, R.B. (1990) Interleukin-1 regulation of procollagenase mRNA and protein in periodontal fibroblasts *in vitro*. *Journal of Periodontal Research*, **25**, 222-229.

Rifkin, B.R. & Heijl, L. (1979) The occurrence of mononuclear cells at sites of osteoclastic bone resorption in experimental periodontitis. *Journal of Periodontology*, **50**, 636-640.

Rohde, D., Schlüter-Wigger, W., Mielke, V., von den Driesch, P., von Gaudecker, B. & Sterry, W. (1992) Infiltration of both T cells and neutrophils in the skin is accompanied by the expression of endothelial leukocyte adhesion molecule-1 (ELAM-1): An immunohistochemical and ultrastructural study. *Journal of Investigative Dermatology*, **98**, 794-799.

Romani, N., Lenz, A., Glassel, H., *et al.* (1989) Cultured human Langerhans cells resemble lymphoid dendritic cells in phenotype and function. *Journal of Investigative Dermatology*, **93**, 600-609.

Roska, A.K., Geppert, T.D. & Lipsky, P.E. (1984) immunoregulation by vascular endothelial cells. *Immunobiology*, **168**, 470-482.

Rossomando, E.F., Kennedy, J.E. & Hadjimichael, J. (1990) Tumour necrosis factor alpha in gingival crevicular fluid as a possible indicator of periodontal disease in humans. *Archives of Oral Biology*, **35**, 431-434.

Rothlein, R., Dustin, M.L., Marlin, S.D. & Springer, T.A. (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *The Journal of Immunology*, **137**, 1270-1274.

Rowden, G. (1981) The Langerhans cell. *C.R.C. Critical Reviews in Immunology*, **3**, 95-180.

Rowden, G., Lewis, M.G. & Sullivan, A.K. (1977) Ia antigen expression on human epidermal Langerhans cells. *Nature*, **268**, 247-248.

Russell, A.L. (1956) A system of classification and scoring for prevalence surveys of periodontal disease. *Journal of Dental Research*, **35**, 350-359.

Russell, A.L. (1967) Epidemiology of periodontal disease. *The International Dental Journal*, **17**, 282-296.

Rustin, M.H.A., Kunaver, M., Cambridge, G., Haskard, D.O. & Dowd, P.M. (1990) Lymphocyte subgroups and expression of endothelial cell adhesion molecules in the skin of patients with systemic sclerosis. Abstracts **94** April 1990 pp 573.

Ryder, M.I. (1980a) Histological and ultrastructural characteristics of the periodontal syndrome in rice rat. I General light microscopic observations and ultrastructural observations of initial inflammatory changes. *Journal of Periodontal Research*, **15**, 502-515.

Ryder, M.I. (1980b) Histological and ultrastructural characteristics of the periodontal syndrome in the rice rat II. Ultrastructural observations on changes in the gingival sulcus, gingival epithelium and lamina propria. *Journal of Periodontal Research*, **15**, 574-584.

Ryder, M.I. (1980c) Histological and ultrastructural characteristics of the periodontal syndrome in the rice rat III. Ultrastructural observations on changes in the transseptal fiber area and alveolar crest. *Journal of Periodontal Research*, **15**, 585-598.

Rylander, H., Attström, R. & Lindhe, J. (1975) Influence of experimental neutropenia in dogs with chronic gingivitis. *Journal of Periodontal Research*, **10**, 315-323.

Sabatini, M., Boyce, B., Aufdemorte, T., Bonewald, L. & Mundy, G.R. (1988) Infusions of recombinant human interleukins 1α and 1β cause hypercalcemia in normal mice. *Proceedings of the National Academy of Sciences of the U.S.A.*, **85**, 5235-5239.

Saglie, F.R., Pertuiset, J., Rezende, M.T., Nestor, M., Marfany, A. & Cheng, J. (1988) *In situ* correlative immuno-identification of mononuclear infiltrates and invasive bacteria in diseased gingiva. *Journal of Periodontology*, **59**, 688-696.

Salmon, M., Kitas, G.D. & Bacon, P.A. (1989) Production of lymphokine mRNA by $CD45RA^+$ and $CD45RA^-$ helper T cells from human peripheral blood and by human $CD4^+$ T cell clones. *The Journal of Immunology*, **143**, 907-912.

Salmon, M., Kitas, G.D., Gasten, J.S.H. & Bacon, P.A. (1988) Interleukin-2 production and response by helper T-cell subsets in man. *Immunology*, **65**, 81-85.

Samant, A., Malik, C.P., Chabra, S.K. & Devi, P.K. (1976) Gingivitis and periodontal disease in pregnancy. *Journal of Periodontology*, **47**, 415-418.

Sanchez-Madrid, F., Krensky, A.M., Ware, C.F. *et al.* (1982) Three distinct antigens associated with human T lymphocyte mediated cytolysis: LFA-1, LFA-2 and LFA-3. *Proceedings of the National Academy of Sciences of the U.S.A.*, **79**, 7489-7493.

Sanders, M.E., Makgoba, M.W., June, C.H., Young, H.A. & Shaw, S. (1989) Enhanced responsiveness of human memory T cells to CD2 and CD3 receptor-mediated activation. *European Journal of Immunology*, **19**, 803-808.

Sanders, M.E., Makgoba, M.W., Sharrow, S.O., *et al.* (1988) Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2 and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-γ production. *The Journal of Immunology*, **140**, 1401-1407.

Savage, N.W., Walsh, L.J. & Seymour, G.J. (1987) Expression of Class I and Class II major histocompatibility complex antigens on oral mucosal epithelium. *Journal of Oral Pathology*, **16**, 153-157.

Saxe, S.R., Greene, J.C., Bohannan, H.M. & Vermillion, J.R. (1967) Oral debris, calculus and periodontal disease in the beagle dog. *Periodontics*, **5**, 217-225.

Saxen, L. (1980) Heredity of juvenile periodontitis. *Journal of Clinical Periodontology*, **7**, 276-288.

Saxen, L. & Nevanlinna, H.R. (1984) Autosomal recessive inheritance of juvenile periodontitis: test of a hypothesis. *Clinical Genetics*, **25**, 332-335.

Schectman, L.R., Ammons, W.F., Simpson, D.M. & Page, R.C. (1972) Host tissue response in chronic periodontal disease II. Histologic features of the normal periodontium, and histopathologic and ultrastructural manifestations of disease in the marmoset. *Journal of Periodontal Research*, **7**, 195-212.

Schenkein, H.A., Best, A.M. & Gunsolley, J.C. (1991) Influence of race and periodontal clinical status on neutrophil chemotactic responses. *Journal of Periodontal Research*, **26**, 272-275.

Schenkein, H.A. & Genco, R.J. (1977a) Gingival fluid and serum in periodontal diseases. I. Quantitative study of immunoglobulins, complement components, and other plasma proteins. *Journal of Periodontology*, **48**, 773-777.

Schenkein, H.A. & Genco, R.J. (1977b) Gingival fluid and serum in periodontal diseases. II. Evidence for cleavage of complement components C3, C3 proactivator (Factor B) and C4 in gingival fluid. *Journal of Periodontology*, **48**, 778-784.

Schiött, C.R. & Löe, H. (1970) The origin and variation in number of leukocytes in the human saliva. *Journal of Periodontal Research*, **5**, 36-41.

Schleimer, R.P. & Rutledge, B.K. (1986) Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with Interleukin 1, endotoxin and tumor-promoting phorbol diesters. *The Journal of Immunology*, **136**, 649-654.

Schonfeld, S.E. & Kagan, J.M. (1982) Specificity of gingival plasma cells for bacterial somatic antigens. *Journal of Periodontal Research*, **17**, 60-69.

Schroeder, H.E. (1969) Melanin containing organelles in cells of the human gingiva. I. Epithelial melanocytes. *Journal of Periodontal Research*, **4**, 1-18.

Schroeder, H.E. (1970a) Quantitative parameters of early human gingival inflammation. *Archives of Oral Biology*, **15**, 383-400.

Schroeder, H.E. (1970b) The structure and relationship of plaque to the hard and soft tissues: Electron Microscopic interpretation. *The International Dental Journal*, **20**, 353-381.

Schroeder, H.E. (1981) Differentiation of Human Oral Stratified Epithelia. New York: S. Karger.

Schroeder, H.E. & Attström, R. (1979) Effect of mechanical plaque control on development of subgingival plaque and initial gingivitis in neutropenic dogs. *Scandinavian Journal of Dental Research*, **87**, 279-287.

Schroeder, H.E. & Graf-De Beer, M. (1976) Stereologic analysis of chronic lymphoid cell infiltrates in human gingiva. *Archives of Oral Biology*, **21**, 527-537.

Schroeder, H.E., Graf-De Beer, M. & Attström, R. (1975) Initial gingivitis in dogs. *Journal of Periodontal Research*, **10**, 128-142.

Schroeder, H.E. & Lindhe, J. (1975) Conversion of stable established gingivitis in the dog into destructive periodontitis. *Archives of Oral Biology*, **20**, 775-782.

Schroeder, H.E., Münzel-Pedrazzoli, S. & Page, R.C. (1973) Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man. *Archives of Oral Biology*, **18**, 899-923.

Schroeder, H.E. & Page, R.C. (1972) Lymphocyte-fibroblast interaction in the pathogenesis of inflammatory gingival disease. *Experientia*, **28**, 1228-1230.

Schroeder, H.E., Rossinsky, K. & Listgarten, M.A. (1989) Human junctional epithelium as a pathway for inflammatory exudation. *Journal de Biologie Buccale*, **17**, 147-157.

Schroeder, H.E. & Theilade, J. (1966) Electron microscopy of normal human gingival epithelium. *Journal of Periodontal Research*, **1**, 95-119.

Scully, C. & Challacombe, S.J. (1979) The migration of ^{111}indium-labelled polymorphonuclear leucocytes into the oral cavity in the rhesus monkey. *Journal of Periodontal Research*, **14**, 475-481.

Serra, H.M., Krowka, J.F., Ledbetter, J.A. & Pilarski, L.M. (1988) Loss of CD45R (Lp220) represents a post-thymic T cell differentiation event. *The Journal of Immunology*, **140**, 1435-1441.

Seymour, G.J. (1987) Possible mechanisms involved in the immunoregulation of chronic inflammatory periodontal disease. *Journal of Dental Research*, **66**, 2-9.

Seymour, G.J., Crouch, M.S., Powell, R.N., *et al.* (1982) The identification of lymphoid cell subpopulations in sections of human lymphoid tissue and gingivitis in children using monoclonal antibodies. *Journal of Periodontal Research*, **17**, 247-256.

Seymour, G.J., Gemmell, E., Walsh, L.J. & Powell, R.N. (1988) Immunohistological analysis of experimental gingivitis in humans. *Clinical and Experimental Immunology*, **71**, 132-137.

Seymour, G.J., Powell, R.N. & Aitken, J.F. (1983a) Experimental gingivitis in humans. A clinical and histologic investigation. *Journal of Periodontology*, **54**, 522-528.

Seymour, G.J., Powell, R.N., Cole, K.L., *et al.* (1983b) Experimental gingivitis in humans. A histochemical and immunological characterization of the lymphoid cell subpopulations. *Journal of Periodontal Research*, **18**, 375-385.

Seymour, G.J., Powell, R.N. & Davis, W.I.R (1979) Conversion of a stable T-cell lesions to a progressive B-cell lesion in the pathogenesis of chronic inflammatory periodontal disease: an hypothesis. *Journal of Clinical Periodontology*, **6**, 267-277.

Seymour, R.A. & Heasman, P.A. (1988) Drugs and the periodontium. *Journal of Clinical Periodontology*, **15**, 1-16.

Shelton, L. & Hall, W. (1968) Human gingival mast cells. *Journal of Periodontal Research*, **3**, 214-223.

Shimizu, Y., Shaw, S., Graber, N., *et al.* (1991) Activation-independent binding of human memory T cells to the adhesion molecule ELAM-1. *Nature*, **349**, 799-802.

Shklar, G., Meyer, I. & Zacarian, S. (1969) Oral lesions in bullous pemphigoid. *Archives of Dermatology*, **99**, 663-670.

Sigusch, B., Klinger, G., Holtz, H. & Süss, J. (1992) *In vitro* phagocytosis by crevicular phagocytes in various forms of periodontitis. *Journal of Periodontology*, **63**, 496-501.

Silberberg-Sinakin, I., Gigli, I., Baer, R.L. & Thorbecke, G.J. (1980) Langerhans cells: Role in contact hypersensitivity and relationship to lymphoid dendritic cells and to macrophages. *Immunological Reviews*, **53**, 203-232.

Silness, P. & Löe, H. (1964) Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. *Acta Odontologica Scandinavica*, **22**, 121-135.

Silver, J.G., Southcott, R.J. & Wade, A.B. (1974) Acute necrotizing ulcerative gingivitis - an evaluation of the ulcer improvement index. *Journal of Periodontology*, **45**, 308-311.

Simpson, D.M. & Avery, B.E. (1973) Pathologically altered fibroblasts within lymphoid cell infiltrates in early gingivitis. *Journal of Dental Research*, **52**, pp 1156.

Simpson, D.M. & Avery, B.E. (1974) Histopathologic and ultrastructural features of inflamed gingiva in the baboon. *Journal of Periodontology*, **45**, 500-510.

Singer, K.H., Denning, S.M., Whichard, L.P. & Haynes, B.F. (1990) Thymocyte LFA-1 and thymic epithelial cell ICAM-1 molecules mediate binding of activated human thymocytes to thymic epithelial cells. *The Journal of Immunology*, **144**, 2931-2939.

Skapski, H. & Lehner, T. (1976) A crevicular washing method for investigating immune components of crevicular fluid in man. *Journal of Periodontal Research*, **11**, 19-24.

Smith, S.H., Brown, M.H., Rowe, D., Callard, R.E. & Beverley, P.C.L. (1986) Functional subsets of human helper-inducer cells defined by a new monoclonal antibody, UCHL1. *Immunology*, **58**, 63-70.

Smith, D.J., Gadalla, L.M., Ebersole, J.L. & Taubman, M.A. (1985) Gingival crevicular fluid antibody levels to oral microorganisms. III. Association of gingival homogenate and gingival crevicular fluid antibody levels. *Journal of Periodontal Research*, **20**, 357-367.

Socransky, S.S. (1977) Microbiology of periodontal disease. Present status and future considerations. *Journal of Periodontology*, **48**, 497-504.

Socransky, S.S., Haffajee, A.D., Goodson, J.M. & Lindhe, J. (1984) New concepts of destructive periodontal disease. *Journal of Clinical Periodontology*, **11**, 21-32.

Spektor, M.D., Vandesteen, G.E. & Page, R.C. (1985) Clinical studies of one family manifesting rapidly progressive, juvenile and prepubertal periodontitis. *Journal of Periodontology*, **56**, 93-101.

Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature*, **346**, 425-434.

Springer, T.A., Davignon, D., Ho, M.-K., Kürzinger, K., Martz, E. & Sanchez-Madrid, F. (1982) LFA-1 and Lyt-2,3, molecules associated with T lymphocyte-mediated killing; and MAC-1, an LFA-1 homologue associated with complement receptor function. *Immunological Reviews*, **68**, 171-195.

Stashenko, P., Dewhirst, F.E., Peros, W.J., Kent, R.L. & Ago, J.M. (1987) Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. *The Journal of Immunology*, **138**, 1464-1468.

Stashenko, P., Fujiyoshi, P., Obernesser, M.S., Prostak, L., Haffajee, A.D. & Socransky, S.S. (1991a) Levels of interleukin 1β in tissue from sites of active periodontal disease. *Journal of Clinical Periodontology*, **18**, 548-554.

Stashenko, P., Jandinski, J.J., Fujiyoshi, P., Rynar, J. & Socransky, S.S. (1991b) Tissue levels of bone resorptive cytokines in periodontal disease. *Journal of Periodontology*, **62**, 504-509.

Stashenko, P., Resmini, M., Haffajee, A.D. & Socransky, S.S. (1985) Helper and suppressor T cells in periodontal disease. *Journal of Periodontal Research*, **20**, 515-521.

Stastny, P., Rosenthal, M., Andreis, M. & Ziff, M. (1975) Lymphokines in the rheumatoid joint. *Arthritis Rheumatism*, **18**, 237-243.

Staunton, D.E., Dustin, M.L. & Springer, T.A. (1989) Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature*, **339**, 61-64.

Steffensen, B., Duong, A.H., Milam, S.B., *et al.* (1992) Immunohistological localization of cell adhesion proteins and integrins in the periodontium. *Journal of Periodontology*, **63**, 584-592.

Steinberg, A.D., Joseph, C.E. & Evans, M.A. (1981) Sulcular penetration and diffusion into surrounding tissues of ^{14}C-Phenytoin and ^{14}C-albumin. *Journal of Periodontal Research*, **16**, 481-489.

Stern, I.B. (1965) Electron microscopic observations of oral epithelium. I. Basal cells and the basement membrane. *Periodontics*, **3**, 224-238.

Stingl, G., Wolff-Schreiner, E.C., Pichler, W.J., Gschnait, F. & Knapp, W. (1977) Epidermal Langerhans cells bear Fc and C3 receptors. *Nature*, **268**, 245-246.

Stoolman, L.M. (1989) Adhesion molecules controlling lymphocyte migration. *Cell*, **56**, 907-910.

Stoufi, E.D., Taubman, M.A., Ebersole, J.L., Smith, D.J. & Stashenko, P.P. (1987) Phenotypic analyses of mononuclear cells recovered from healthy and diseased human periodontal tissues. *Journal of Clinical Immunology*, **7**, 235-245.

Streuli, M., Morimoto, C., Schrieber, M., Schlossman, S.F. & Saito, H. (1988) Characterization of CD45 and CD45R monoclonal antibodies using transfected mouse cell lines that express individual human leukocyte common antigens. *The Journal of Immunology*, **141**, 3910-3914.

Suomi, J.D., Greene, J.C., Vermillion, J.R., Chang, J.J. & Leatherwood, E.C. (1969) The effect of controlled oral hygiene procedures on the progression of periodontal disease in adults: Results after two years. *Journal of Periodontology*, **40**, 416-420.

Suomi, J.D., Greene, J.C., Vermillion, J.R., Doyle, J., Chang, J.J. & Leatherwood, E.C. (1971a) The effect of controlled oral hygiene procedures on the progression of periodontal disease in adults: Results after third and final year. *Journal of Periodontology*, **42**, 152-160.

Suomi, J.D., Leatherwood, E.C. & Chang, J.J. (1973) A follow-up study of former participants in a controlled oral hygiene study. *Journal of Periodontology*, **44**, 662-666.

Suomi, J.D., Smith, L.W. & McClendon, B.J. (1971b) Marginal gingivitis during a sixteen-week period. *Journal of Periodontology*, **42**, 268-270.

Sutcliffe, P. (1972) A longitudinal study of gingivitis and puberty. *Journal of Periodontal Research*, **7**, 52-58.

Suzuki, J.B., Collison, B.C., Falker, W.A.Jr. & Nauman, R.K. (1984) Immunologic profile of juvenile periodontitis. II. Neutrophil chemotaxis, phagocytosis, and spore germination. *Journal of Periodontology*, **55**, 461-467.

Symington, F.W., Hedges, D.L. & Hakomori, S.-I. (1985) Glycolipid antigens of human polymorphonuclear neutrophils and the inducible HL-60 myeloid leukemia line. *The Journal of Immunology*, **134**, 2498-2506.

Taichman, N.S., Freedman, H.L. & Uriuhara, T. (1966) Inflammation and tissue injury - I The response to intradermal injections of human dento-gingival plaque in normal and leukopenic rabbits. *Archives of Oral Biology*, **11**, 1385-1392.

Taichman, N.S., Tsai, C.C., Shenker, B.J. & Boehringer, H. (1984) Neutrophil interactions with oral bacteria as a pathogenic mechanism in periodontal diseases. In G. Weissmann ed. *Advances in Inflammation Research*, pp113-142, New York: Raven Press.

Takada, H., Mihara, J., Moisaki, I. & Hamada, S. (1991) Induction of Interleukin-1 and -6 in human gingival fibroblast cultures stimulated with Bacteroides lipopolysaccharides. *Infection and Immunity*, **59**, 295-301.

Tedder, T.F., Clement, L.T. & Cooper, M.D. (1985a) Human lymphocyte differentiation antigens HB-10 and HB-11 I. Ontogeny of antigen expression. *The Journal of Immunology*, **134**, 2983-2988.

Tedder, T.F., Cooper, M.D. & Clement, L.T. (1985b) Human lymphocyte differentiation antigens HB-10 and HB-11 II. Differential production of B cell growth factors and differentiation factors by distinct helper T cell populations. *The Journal of Immunology*, **134**, 2989-2994.

Tew, J.G., Burmeister, J.A., Paccanis, K.G. & Ranney, R.R. (1983) Spontaneous lymphocyte proliferation and the periodontal status of young adults. *Journal of Periodontal Research*, **18**, 354-540.

Tew, J.G., Marshall, D.R., Burmeister, J.A. & Ranney, R.R. (1985) Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. *Infection and Immunity*, **49**, 487-493.

Tew, J.G., Miller, G.A., Greene, E.J., *et al.* (1981) Immunological studies of young adults with severe periodontitis. II. Cellular factors. *Journal of Periodontal Research*, **16**, 403-416.

Thilander, H. (1964) Permeability of the gingival pocket epithelium. *The International Dental Journal*, **14**, 416-421.

Thorbecke, G.J., Silberberg-Sinakin, I. & Flotte, T.J. (1980) Langerhans cells as macrophages in skin and lymphoid organs. *Journal of Investigative Dermatology*, **75**, 32-43.

Thornhill, M.H. & Haskard, D.O. (1990) IL-4 regulates endothelial cell activation by Il-1, tumor necrosis factor, or IFN-γ. *The Journal of Immunology*, **145**, 865-872.

Thornhill, M.H., Wellicome, S.M., Mahiouz, D.L., Lanchbury, J.S.S., Kyan-Aung, U. & Haskard, D.O. (1991) Tumor necrosis factor combines with IL-4 or IFN-γ to selectively enhance endothelial cell adhesiveness for T cells. The contribution of vascular cell adhesion molecule-1-dependent and --- independent binding mechanisms. *The Journal of Immunolgy*, **146**, 592-598.

Thurre, C., Robert, M., Cimasoni, G. & Baehni, P. (1984) Gingival sulcular leukocytes in periodontitis and in experimental gingivitis in humans. *Journal of Periodontal Research*, **19**, 457-468.

Topoll, H.H., Zwadlo, G., Lange, D.E. & Sorg, G. (1989) Phenotypic dynamics of macrophage subpopulations during human experimental gingivitis. *Journal of Periodontal Research*, **24**, 106-112.

Trefzer, U., Brockhaus, M., Loetscher, H., *et al.* (1991) 55-kd tumor necrosis factor receptor is expressed by human keratinocytes and plays a pivotal role in regulation of human keratinocyte ICAm-1 expression. *Journal of Investigative Dermatology*, **97**, 911-916.

Van der Waal, I. (1991) Non-plaque related periodontal lesions. An overview of some common and uncommon lesions. *Journal of Clinical Periodontology*, **18**, 436-440.

Van Dyke, T.E., Horoszewicz, H.U., Cianciola, L.J. & Genco, R.J. (1980) Neutrophil chemotaxis dysfunction in human periodontitis. *Infection and Immunity*, **27**, 124-132.

Van Dyke, T.E., Levine, M.J. & Genco, R.J. (1985) Neutrophil function and oral disease. *Journal of Oral Pathology*, **14**, 95-120.

Van Dyke, T.E., Offenbacher, S., Place, D., Dowell, V.R. & Jones, J. (1988) Refractory periodontitis: mixed infection with *Bacteriodes gingivalis* and other unusual *Bacteriodes* species. A case report. *Journal of Periodontology*, **59**, 184-189.

Van Dyke, T.E., Schweinebraten, M., Cianciola, L.J., Offenbacher, S. & Genco, R.J. (1985) Neutrophil chemotaxis in families with localized juvenile periodontitis. *Journal of Periodontal Research*, **20**, 503-514.

Van Loon, L.A.J., Krieg, S.R., Davidson, C.L. & Bos, J.D. (1989) Quantification and distribution of lymphocyte subsets and Langerhans cells in normal human oral mucosa and skin. *Journal of Oral Pathology and Medicine*, **18**, 197-201.

van Palenstein-Helderman, W.D. (1975) Total viable count and differential count of vibrio (campylobacter) sputorum, fusobacterium nucleatum, selenomonas sputigena, bacteroides ochraceus and veillonella in the inflamed and non inflamed human gingival crevice. *Journal of Periodontal Research*, **10**, 294-305.

Waerhaug, J. (1977) Subgingival plaque and loss of attachment in periodontosis as evaluated on extracted teeth. *Journal of Periodontology*, **48**, 125-130.

Wagner, C.R., Vetto, R.M. & Burger, D.R. (1984) The mechanism of antigen presentation by endothelial cells. *Immunobiology*, **168**, 453-469.

Waldrop, T.C., Anderson, D.C., Hallmon, W.W., Schmalstieg, F.C. & Jacobs, R.L. (1987) Periodontal manifestations of the heritable Mac-1, LFA-1, deficiency syndrome. Clinical, histopathologic and molecular characteristics. *Journal of Periodontology*, **58**, 400-416.

Wallace, D.L. & Beverley, P.C.L. (1990) Phenotypic changes associated with activation of $CD45RA^+$ and $CD45RO^+$ T cells. *Immunology*, **69**, 460-467.

Walker, S.M. & Lucas, Z.J. (1972) Cytotoxic activity of lymphocytes. II. Studies on mechanism of lymphotoxin-mediated cytotoxicity. *The Journal of Immunology*, **109**, 1233-1244.

Walsh, L.J., Armitt, K.L., Seymour, G.J. & Powell, R.N. (1987a) The immunohistology of chronic gingivitis in children. *Paediatric Dentistry*, **9**, 26-32.

Walsh, L.J., Savage, N.W., Ishii, T. & Seymour, G.J. (1990a) Immunopathogenesis of oral lichen planus. *Journal of Oral Pathology and Medicine*, **19**,389-396.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1985) The *in vitro* effect of retinol on human gingival epithelium. II Modulation of Langerhans cell markers and interleukin-1 production. *Journal of Investigative Dermatology*, **85**, 501-506.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1986a) Modulation of Class II (DR and DQ) antigen expression on gingival Langerhans cells *in vitro* by gamma interferon and prostaglandin E2. *Journal of Oral Pathology*, **15**, 347-351.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1986b) Modulation of gingival Langerhans cells T6 antigen expression *in vitro* by interleukin 1 and an interleukin-1 inhibitor. *Clinical and Experimental Immunology*, **64**, 334-341.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1987b) Differential expression of Class II(DR & DQ) antigens by human gingival Langerhans cells and keratinocytes *in vitro*. *Journal of Oral Pathology*, **16**, 27-30.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1988) The regulation of Langerhans cell T6, DR and DQ antigen expression: an hypothesis. *Journal of Oral Pathology*, **17**, 43-46.

Walsh, L.J., Seymour, G.J. & Powell, R.N. (1990b) Human gingival Langerhans cells stimulate allogeneic lymphocytes: Requirements for MHC class II antigens. *Journal of Periodontology*, **61**, 328-333.

Walsh, L.J., Tseng, P.W., Savage, N.W. & Seymour, G.J. (1989) Expression of CDw29 and CD45R antigens on epithelial cells in oral lichen planus. *Journal of Oral Pathology and Medicine*, **18**, 360-365.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M. & Seed, B. (1990) Recognition by ELAM-1 of the Sialyl-Le^x determinant on myeloid and tumor cells. *Science*, **250**, 1132-1134.

Wantanabe, K., Hagen, K.L. & Andersen, B.R. (1991) CD11b expression on neutrophils in human crevicular fluid collected from clinically healthy gingivae. *Journal of Periodontal Research*, **26**, 91-96.

Weinstein, E., Mandel, I.D., Salkind, A., Oshrain, H.I. & Pappas, G.D. (1967) Studies of gingival fluid. *Periodontics*, **5**, 161-166.

Wellicome, S.M., Thornhill, M.H., Pitzalis, C., *et al.* (1990) A monoclonal antibody that detects a novel antigen on endothelial cells that is induced by tumour necrosis factor, IL-1 or lipopolysaccharide. *The Journal of Immunology*, **144**, 2558-2565.

Wennström, J., Heijl, L., Lindhe, J. & Socransky, S. (1980) Migration of gingival leukocytes mediated by plaque bacteria. *Journal of Periodontal Research*, **15**, 363-372.

Wilde, G., Cooper, M. & Page, R.C. (1977) Host tissue response in chronic periodontal disease VI The role of cell-mediated hypersensitivity. *Journal of Periodontal Research*, **12**, 179-196.

Wilkinson, P.C. & Lackie, J.M. (1979) The adhesion, migration and chemotaxis of leukocytes in inflammation. *Current Topics in Pathology*, **68**, 47-88.

Wilton, J.M.A. (1982) Polymorphonuclear leukocytes of the human gingival crevice: Clinical and experimental studies of cellular function in humans and animals. In *Host-Parasite interactions in periodontal diseases*, ed. Genco, R.J. & Mergenhagen, S.E., Washington DC, USA: pp 246-260.

Wilton, J.M.A. (1984) Future control of dental disease by immunization: Vaccines and oral health. *The International Dental Journal*, **34**, 177-183.

LIST OF PUBLICATIONS

The following publications include material presented as part of this thesis:

Moughal, N.A., Adonogianaki, E., Thornhill, M.H. & Kinane, D.F. (1992) Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and Intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally induced gingivitis. *Journal of Periodontal Research*, **27**, in press.

Moughal, N.A., Adonogianaki, E. & Kinane, D.F. (1992) Langerhans cell dynamics in human gingiva during experimentally induced inflammation. *Journal Biologie de Buccale*, **20**, in press.

Adonogianaki, E., Moughal, N.A. & Kinane, D.F. (1992) Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. *Journal of Clinical Periodontology*, **19**, in press.

Kinane, D.F., Adonogianaki, E., Moughal, N., Winstanley, F.R., Mooney, J. & Thornhill, M. (1991) Immunocytochemical characterization of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during human experimental gingivitis. *Journal of Periodontal Research*, **26**, 286-288.

Kinane, D.F., Winstanley, F.P., Adonogianaki, E. & Moughal, N.A. (1992) Bioassay of interleukin 1 (IL-1) in human gingival crevicular fluid during experimental gingivitis. *Archives of Oral Biology*, **37**, 153-156.

The following publication is indirectly related to this thesis:

Kinane, D.F., Karim, S.N., Garioch, J.J., Al Badri, A.T., Moughal, N. & Goudie, R.B. (1992) Heterogeneity and selective localisation of T cell clones in human skin and gingival mucosa. *Journal of Periodontal Research*, in press.

Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally-induced gingivitis

N. A. Moughal[1], E. Adonogianaki[1], M. H. Thornhill[2] and D. F. Kinane[1]

[1]Department of Oral Medicine and Pathology, Glasgow Dental Hospital & School, Glasgow, [2]Department of Oral Medicine and Periodontology, The London Hospital Medical College, Turner Street, London, U.K.

Moughal NA, Adonogianaki E, Thornhill MH, Kinane DF: Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally-induced gingivitis. J Periodont Res 1992; 27: 00–00.

The changes in vascular adhesion molecule expression and numbers of infiltrating leukocytes during a 21-day experimental gingivitis episode were investigated immunohistochemically. Monoclonal antibodies to ELAM-1 (1.2B6), ICAM-1 (6.5B5), CD3 (OKT3 – pan-T cell) and neutrophils (PMN-elastase) were used to identify positive vessels and leukocytes within gingival biopsies taken on d 0, 7, 14 and 21. Vascular endothelium expressed ELAM-1 and ICAM-1 both in clinically 'healthy' tissue (d 0) and in experimentally inflamed tissue (d 7 to 21). Positive vessels were found mainly in the connective tissue subjacent to the junctional epithelium where the highest numbers of T cells and neutrophils were also seen. Although T cells were found in all tissue areas studied, neutrophils were largely concentrated in the junctional epithelium and the subjacent connective tissue but were absent from the oral epithelial region. As the experimental gingivitis developed, the number of T cells or neutrophils in the different tissue regions did not change significantly although the most intense vascular ICAM-1 and ELAM-1 staining redistributed to the CT adjacent to the junctional epithelium. A prominent feature was the intense ICAM-1-positive staining of the junctional epithelium and its absence in the closely adjacent oral epithelium, in both clinically 'healthy' and inflamed tissue. The gradient of ICAM-1 in junctional epithelium, with the strongest staining on the crevicular aspect plus the vascular expression of ELAM-1 and ICAM-1 in both clinically 'healthy' and inflamed tissue may be crucial processes which direct leukocyte migration towards the gingival crevice.

Key words: adhesion molecules – experimental gingivitis – inflammation – leucocyte infiltration

Accepted for publication 7 February 1992

Introduction

Recruitment of leukocytes into areas of injury or infection is essential for host defence. The constant migration of T cells and other leukocytes to sites throughout the body allows the full repertoire of the immune system to protect the tissues from a variety of antigenic challenges. Leukocyte migration into tissues is particularly prominent during inflammatory responses and results from the cytokine-induced expression of adhesion molecules on the surface of vascular endothelial cells (ECs) (1–7). Endothelial leukocyte adhesion molecule 1 (ELAM-1) (8) and intercellular adhesion molecule 1 (ICAM-1) are two adhesion molecules which appear to be crucial for cellular trafficking.

Although not normally expressed on uninflamed endothelium, ELAM-1 expression may be transiently induced on endothelium in almost any in-

Abbreviations: CT – connective tissue; ELAM-1 – endothelial leukocyte adhesion molecule 1; ICAM-1 – intercellular adhesion molecule 1; JE – junctional epithelium; JE/CT – connective tissue subjacent to junctional epithelium; OE – oral epithelium; OE/CT – connective tissue subjacent to oral epithelium; PMN – polymorphonuclear leukocyte or neutrophil; SE – sulcular epithelium.

flamed tissue: however, expression on inflamed endothelium of skin may be enhanced or prolonged compared to other sites (9). In contrast, ICAM-1 is constitutively expressed on ECs and exhibits no tissue-specific restriction in its expression (3, 10). ICAM-1 expression may be enhanced *in vitro* by the action of cytokines (11–13) and this is reflected *in vivo* by a greater intensity of expression on ECs at sites of inflammation (14–16).

Adhesion molecule expression is induced early during experimental inflammatory responses in the skin of monkeys (16) and humans (17). Similar experiments have not previously been performed in gingival tissue although Crawford and Hopp (1990) (18) have reported ICAM-1 expression in healthy gingival tissue.

The aim of this study was to examine immunohistochemically the changes in T-cell numbers, neutrophil (PMN) numbers and ELAM-1 and ICAM-1 expression during 21 d of experimentally-induced gingivitis.

Material and Methods

Clinical material

Biopsy material was obtained using the experimental gingivitis model of Loe, Theilade and Jensen (1965) (19). Ethical approval was granted by the local ethics committee. Six dental students (5 male, 1 female) who consented to participate in the study received oral hygiene instruction and frequent prophylaxis until maximal clinical gingival health was obtained. All subjects were then instructed to cease all oral hygiene measures and plaque was allowed to accumulate undisturbed for a period of 21 d. The clinical indices were recorded (every 7 d), at each biopsy site, using the plaque index (PI) of Silness and Loe (1964) (20) and the modified gingival index (MGI) of Lobene *et al.* (1986) (21). The MGI is a modification of the Loe and Silness (1963) (22) gingival index (GI); it omits the bleeding-upon-probing component of the GI and permits a non-invasive evaluation of the early visible

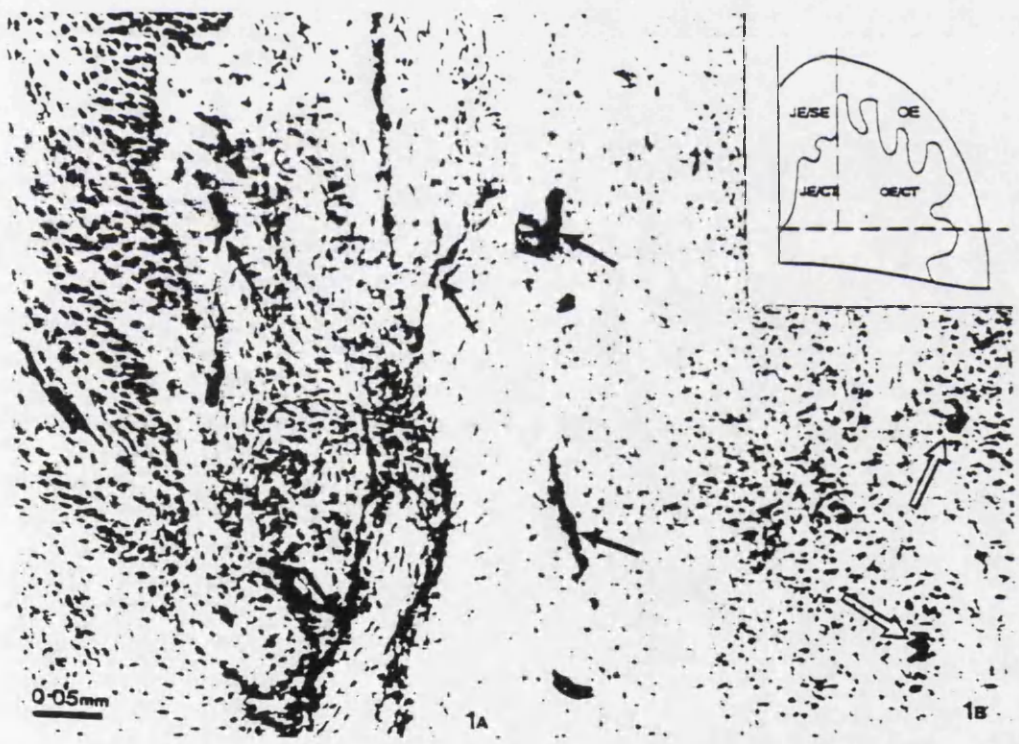

Fig. 1(a). ELAM-1$^+$ vessels in the connective tissue area of a day-0 biopsy (solid arrows); *(b)* A day-21 biopsy with the most intense staining ELAM-1$^+$ vessels located at the JE/CT area (solid arrows), with weaker staining ELAM-1$^+$ vessels located towards the connective tissue (open arrows) (Immunoperoxidase, Magnification 225 x), *(inset)* Areas used to quantify cellular infiltrate. (JE = junctional epithelium; OE = oral epithelium; JE/CT = junctional epithelium subjacent to connective tissue; OE/CT = oral epithelium subjacent to connective tissue).

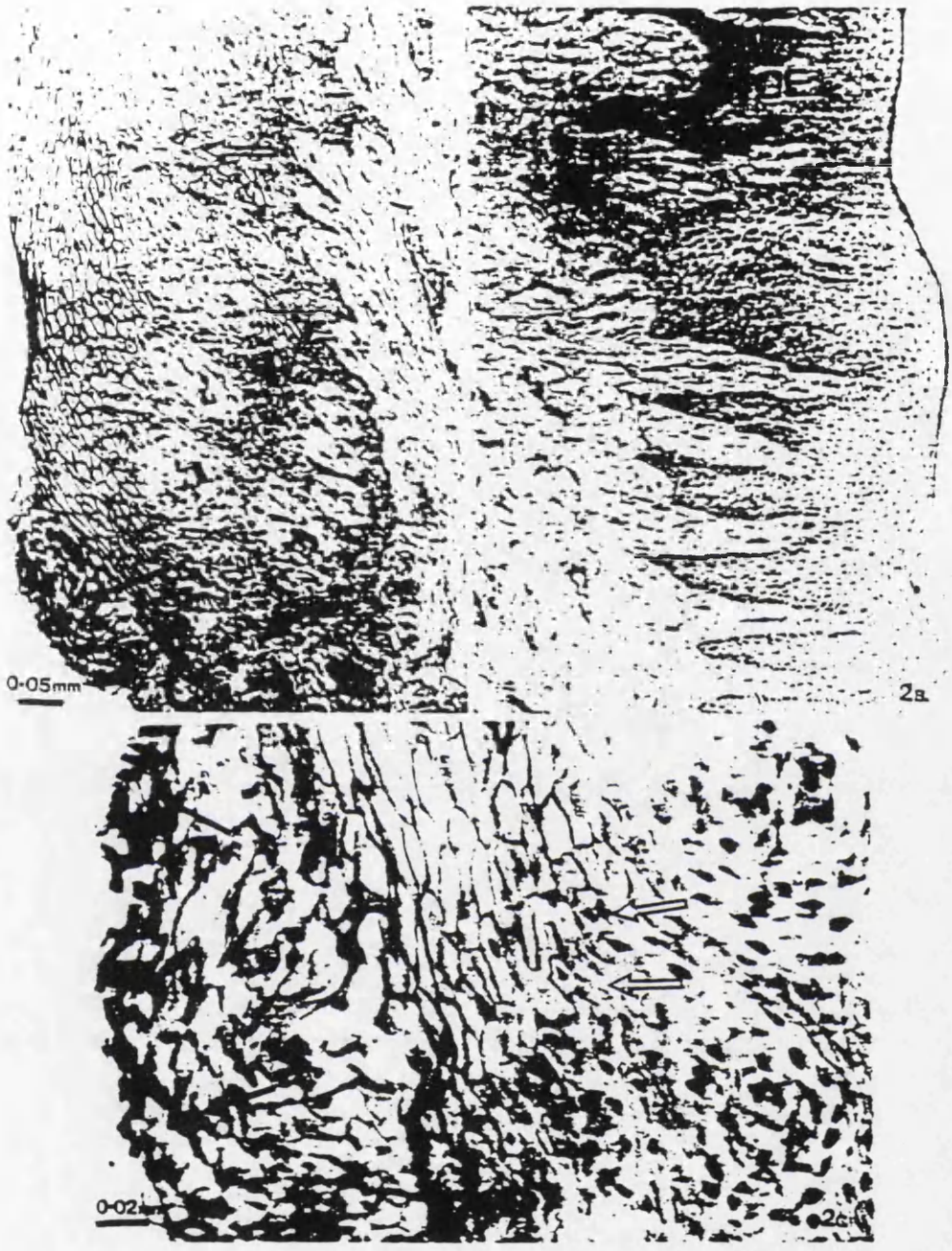

Fig. 2(a). Intense ICAM-1 staining of JE keratinocytes (solid arrow) with moderate staining of SE keratinocytes (open arrow); *(b)* Absence of ICAM-1 staining of OE keratinocytes in clinically healthy tissue (day 0) (Magnification 140 ×); *(c)* Day-7 biopsy showing an ICAM-1 gradient, with maximal staining at the JE/tooth interface (solid arrows), with weaker staining in the basal layers of the JE (open arrows) (Immunoperoxidase. Magnification 462 ×).

Table 1. PMN and T-cell numbers over the 21 days of experimental inflammation. Values shown are means and standard errors (in parenthesis) of the leukocytes per square millimeter within each region

	CD3⁺ T cells				PMNs			
Day	JE/CT	OE/CT	JE	OE	JE/CT	OE/CT	JE	OE
0	1842 (957)	180 (109)	178 (110)	28 (19)	1258 (678)	41 (21)	321 (130)	0 (0)
7	3326 (899)	811 (265)	895 (498)	193 (34)	449 (78)	22 (13)	59 (59)	0 (0)
14	2620 (1044)	285 (52)	736 (347)	108 (30)	874 (347)	0 (0)	ND	0 (0)
21	1846 (748)	554 (184)	978 (174)	59 (30)	924 (348)	27 (13)	684 (361)	0 (0)

ND = not done.

changes in the extent and severity of gingivitis. One examiner was used throughout the experiment to record the clinical indices (E.A.). Gingival biopsies (2 mm × 2 mm) were obtained surgically on d 0 of the experimental gingivitis phase from the mid-buccal aspect of the lower right first permanent molar. The remaining first permanent molars were then biopsied in rotation (d 7, 14 and 21). In total, four biopsies were taken from each individual.

Immunohistochemistry

All tissue biopsies were embedded in Tissue Tek and snap frozen. 8 μm-thick sections were cut and stained using the ABC method of Hsu and Raine (1981) (23). Frozen cryostat sections of the specimens were air-dried and fixed in cold acetone. Following fixation the sections were blocked with horse serum before the addition of the primary monoclonal antibodies. The primary antibodies (mouse anti-human monoclonal antibodies 1.2B6 (anti-ELAM-1, 1:20 dilution), 6.5B5 (anti-ICAM-1, 1:30 dilution), OKT3 (CD3, 1:25 dilution) and PMN-Elastase (1:300 dilution, DAKO, U.K.) were reacted for 1 hour at 20°C. Monoclonal antibodies against ELAM-1 and ICAM-1 were the kind donation of Dr. D. O. Haskard (Royal Postgraduate Medical School, London, U.K.) and anti-CD3 monoclonal antibody supernatant was produced from the OKT3 hybridoma cell line obtained from the American Type Culture Collection. The primary monoclonal antibodies were then labelled with biotinylated anti-mouse immunoglobulin. Before the addition of avidin-biotinylated peroxidase complex (Vector Laboratories, Petersborough, U.K.) the sections were deperoxidized (0.03% hydrogen peroxidase in methanol) to remove endogenous peroxidase. The reaction was developed using the substrate diaminobenzidine tetrahydrochloride and to enhance staining the sections were incubated in 0.5% copper sulphate solution before counterstaining with Mayer's hematoxylin. Between each incubation the slides were washed with phosphate-buffered saline (pH 7.4). Monoclonal antibody specificities were confirmed using human tonsil and human skin as control tissue and appropriate negative control sections treated with non-specific primary antibodies. One section from each biopsy was also stained with hematoxylin and eosin.

Enumeration

Due to clinical and surgical restrictions incumbent in biopsying gingival of teeth which were not extracted, it was difficult to obtain complete apical junctional epithelium (JE); therefore, in order to quantify the biopsy, it was divided into four areas – junctional epithelium/sulcular epithelium (JE/SE), oral epithelium (OE), CT subjacent to the JE (JE/CT) and CT subjacent to the OE (OE/CT) (Fig. 1 inset). A five-point scale (0–4) was used to document the changes in intensity and global extent of antigen expression within the biopsies, with ELAM-1 and ICAM-1 being quantified according to the criteria of Messadi *et al.* (1987) (24) (0, no staining; 1, weak focal granular staining; 2, moderate staining of vessels; 3, strong staining of vessels, and 4, very strong staining of vessels). ICAM-1 graded in the four areas and ELAM-1 graded only in the JE/CT and OE/CT (JE and OE were ELAM-1-negative). T cells and PMNs were quantified as CD3⁺ or PMN-Elastase⁺ cells within the four areas. Positive cells within these four areas were counted 'blind' at ×400 magnification and the area measured from each section using computer-assisted image analysis (Video Vector Dynamics, Glasgow, U.K.) and the results expressed as mean number of positive cells per square millimeter (mm^2) for each biopsy.

Statistical analysis

The Mann-Whitney test was utilised to determine if T cells numbers differed from PMNs numbers within the same area over time. In addition, analy-

Fig. 3. Photomicrographs of *(a)* ELAM-1⁺ vessels (solid arrows); *(b)* PMNs (solid arrows); *(c)* ICAM-1⁺ vessels (solid arrows); and *(d)* CD3⁺ staining (solid arrows) at the JE/CT area within the same day-0 biopsy. (Immunoperoxidase. Magnification 140×).


0·05mm
3A
3B
3C
3D

sis of variance (ANOVA) was used to determine if T cell or PMN numbers changed within any one given area over time. Both tests were carried out using the Minitab statistical package on an IBM PC.

Results

Following withdrawal of oral hygiene procedures the PI at the biopsy sites rose (d 0) (0.17 ± 0.4) and peaked at d 21 (2.2 ± 0.4) with the MGI following closely behind (d 0) (0 ± 0) to d 21 (2.2 ± 0.4) (mean and standard deviation).

ELAM-1$^+$ blood vessels were present in both the JE/CT and OE/CT of healthy gingiva (d 0) (Fig. 1a), although expression was mainly concentrated in the JE/CT area. As the inflammation developed, the concentration and intensity of staining in the JE/CT area became more marked, as demonstrated by a 25% increase in the numbers of biopsies with stronger staining vessels at the JE/CT (d 21 compared with d 0) (Fig. 1b). ICAM-1 staining of blood vessels in the connective tissue also showed a similar distribution and pattern of staining.

Clinically 'healthy' tissue also demonstrated intense ICAM-1 staining of the keratinocytes at the JE, with moderate staining of SE keratinocytes and no staining of OE keratinocytes (Figs. 2a & 2b). With the subsequent development of inflammation there was little change in the intensity or distribution of ICAM-1 staining and no spread of ICAM-1 positivity to the OE keratinocytes. Throughout the 21 d the JE keratinocytes ICAM-1 staining showed a gradient, with the most intense ICAM-1 staining towards the crevicular/tooth aspect and decreasing staining intensity towards the basal layers (Fig. 2c). Apical JE, when present, also expressed ICAM-1. Unpublished observations from within our clinic and laboratories indicate that surgical biopsy tissue from chronically inflamed periodontal sites also exhibits ELAM-1- and ICAM-1-positive vessels.

PMN numbers and T-cell numbers over the 21-d period did not change significantly in the four areas of the gingival biopsy examined (JE, OE, JE/CT and OE/CT) (Table 1), except the increase in T cell numbers as inflammation developed, d 0 to d 7 (ANOVA $p<0.01$). However, there were significantly more PMNs present in the JE/CT than the OE/CT at all time points (Mann-Whitney $p<0.05$ in each case), whereas T-cell numbers were only elevated significantly at d 14 (Mann-Whitney $p<0.05$) and this corresponded to the distribution of ICAM-1- and ELAM-1-positive blood vessels. Interestingly, in the connective tissue CD3$^+$ T cells showed a tendency to cluster around the tips of the oral epithelial papillae and this was a feature of biopsies taken at all time points. In the epithelium, T cells were noted both in the OE and the JE although by d 21 the numbers of T cells in the JE were significantly higher than the number in the OE ($p<0.05$). In contrast, PMNs were not observed in the OE but were present at all time points in the JE. Furthermore, within the JE PMNs were found to be concentrated towards the crevicular/tooth aspect, where adhesion molecule staining was most intense. Generally the greatest concentration of T cells and PMNs in the JE/CT and JE area coincided with the location of the most intense ELAM-1 and ICAM-1 staining (Figs. 3a–3d).

With the exception of the OE where a few T cells but no PMNs were present, there was no significant difference in the number of T cells compared to the number of PMNs in the different tissues compartments at d zero. However, by d 7, as inflammation developed, there were significantly more T cells than PMNs present in each of the tissue compartments ($p<0.05$ in each case), although by d 14 and 21 these differences were still only significant in the OE and OE/CT (Table 2). Differences in T-cell and PMN number, and in ELAM-1 and ICAM-1 intensity between subjects were noted during the 21 d of experimentally-induced gingivitis. These interindividual variations, however, were not statistically significant and the overall changes were similar for all individuals.

Table 2. PMN:T-cell ratios during 21 days of experimentally-induced gingival inflammation. Values shown are means and standard errors (in parenthesis) of the leukocytes per square millimeter within each region

	Ratios	T cells and PMNs compared in			
Day	p value	JE/CT	OE/CT	JE	OE
0	PMN:T	1:1	1:4	1:1	0:28
	p value	NS	NS	NS	<0.001
7	PMN:T	1:7	1:37	1:15	0:193
	p value	<0.05	<0.005	<0.05	<0.001
14	PMN:T	1:3	0:285	ND	0:108
	p value	NS	<0.001		<0.001
21	PMN:T	1:2	1:21	1:1	0:59
	p value	NS	<0.05	NS	<0.001

ND = not done.

Discussion

This study demonstrated the presence of adhesion molecules in clinically healthy and in experimentally-inflamed gingival tissue, whereas previous studies have shown that normal 'uninflamed' tissue is negative for ELAM-1 (16, 17, 25, 26). *In vitro* studies have shown that ELAM-1 acts as a highly specific endothelial cell ligand for the binding of peripheral blood PMNs (8, 27) and *in vivo* studies

have confirmed a strong correlation between ELAM-1 expression on blood vessels and the accumulation of PMNs in the tissues during experimental inflammatory responses (16, 17, 25). The intensity of ELAM-1 and ICAM-1 staining of the blood vessels was high at each time point with the strongest expression of these molecules occurrind in the same areas (JE and JE/CT) where PMN and T cells were located in greatest abundance (Fig. 3a–3d). Seymour *et al.* (28,29) comment on the sparse T-lymphocyte infiltrate within 7 to 10 d of experimental gingivitis. The immunohistochemistry technique employed in the present study is very specific and sensitive and accordingly T cell numbers were found in greater numbers than with previous techniques. The greater presence of ELAM-1 and ICAM-1 at the JE and JE/CT area compared with OE may influence and explain the increased leukocyte traffic (PMN and T cells) in these areas, resulting in local defence of the periodontal tissue.

Although no significant difference was detected in the number of PMNs in the JE/SE or JE/CT areas at different time points, this does not mean that the number of PMNs trafficking through the tissues does not increase during the course of experimental gingivitis. It simply reflects the difficulty of trying to assess the dynamic flow of cells into and out of the tissues by counting those caught in the middle at one particular moment in time. In other words if the recruitment of PMNs into the tissues is balanced by their loss into the gingival crevice there may be little change in PMN numbers in the tissues even if there is an increase in the rate of traffic over the 21 d (Table 1). The fact that ELAM-1 expression was detected throughout the 21 d of experimentally-induced inflammation suggests one of two possibilities: either the blood vessels in gingiva are functionally specialized and constitutively express ELAM-1 plus high levels of ICAM-1 to facilitate leukocyte traffic into the gingival crevice; or, the gingival vessels are permanently activated even in 'health' due perhaps to the constant ingress of bacterial products such as LPS across the junctional epithelium. Further support for the idea that gingival post-capillary venules may be specialized to express ELAM-1 constitutively, is provided by the fact that they do not exhibit the normal modulation of ELAM-1 expression seen in other situations (16, 17, 25).

ICAM-1 has been shown to play a role in the adhesion of PMNs and T cells to ECs and it is also important in facilitating the trans-endothelial cell migration of leukocytes (3, 10, 30). ICAM-1 expression on the blood vessels changed very little in relation to its distribution throughout the 21 d and this was reflected in the T-cell numbers not changing within the different tissue compartments (Table 1). It was noted that junctional epithelium, and to a lesser extent sulcular epithelium, remained strongly positive for ICAM-1 throughout the 21 d of experimental gingivitis. At no point was ICAM-1 positivity of the adjacent oral epithelium noted and the transition from ICAM-1-positive junctional and sulcular epithelium to oral epithelium was always quite distinct and marked (Fig. 2a & 2b). The intensity of the ICAM-1 staining at JE is greater than is often seen in other epithelial tissues even in the presence of an immune-mediated inflammatory response or direct cytokine stimulation. Taken together, these observations suggest that junctional epithelium may be specially adapted to constitutively express ICAM-1. Since ICAM-1 expression on keratinocytes facilitates leukocyte-keratinocyte adhesion and transmigration (30, 31) this adaption may help to explain the special ability of junctional epithelium to permit leukocyte traffic into the gingival crevice. Unlike endothelial cells, keratinocytes do not normally express ICAM-1; however, keratinocyte ICAM-1 expression can be induced both *in vivo* and *in vitro* by the action of tumor-necrosis factor or interferon-γ, but not by interleukin-1 or LPS (14, 32, 33).

The importance of these mechanisms in protecting against periodontal disease is highlighted by the rapid and severe periodontitis that characterizes leukocyte adhesion deficiency (34–36). It is important that the kinetics of adhesion molecule expression and the dynamics of leukocyte trafficking are studied in greater detail in order to ascertain if defects in adhesion molecule expression play a part in determining an individuals susceptibility to periodontal disease.

Acknowledgments

The authors would like to thank Professor D. G. MacDonald for his help with the enumeration procedures and technical skill in photography. This work was in part supported by a grant from the Glasgow University Medical Research Funds Committee.

References

1. Cavender DE, Haskard DO, Joseph B, Ziff M. Interleukin 1 increases the binding of human B and T lymphocytes to endothelial cell monolayers. *J Immunol* 1986; 136: 203–207.
2. Cavender DE, Haskard DO, Maliakkal D, Ziff M. Separation and characterization of human T lymphocytes with varying adhesiveness for endothelial cells. *Cell Immunol* 1988; 117: 111–126.
3. Dustin ML, Rothelin R, Bhan AK, Dinarello CA, Springer TA. Induction by IL 1 and interferon-γ: Tissue distribution, Biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol* 1986; 137: 245–254.
4. Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA. Stim-

ulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumour necrosis factor. *Proc Natl Acad Sci USA* 1985; 82: 8667–8671.
5. Haskard DO, Cavender D, Fleck RM, Sontheimer R, Ziff M. Human dermal microvascular endothelial cells behave like umbilical vein endothelial cells in T-cell adhesion studies. *J Invest Dermatol* 1987; 88: 340–344.
6. Schleimer RP, Rutledge BK. Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with Interleukin 1, endotoxin and tumour-promoting phorbol diesters. *J Immunol* 1986; 136: 649–654.
7. Pober JS, Bevilacqua MP, Mendrick DL, Lapierre LA, Fiers W, Gimbrone MA Jr. Two distinct monokines, Interleukin 1 and Tumour Necrosis Factor, each independently induce biosynthesis and transient expression of the same antigen on the surface of cultured human vascular endothelial cells. *J Immunol* 1986; 136: 1680–1687.
8. Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA Jr. Identification of an inducible endothelial-leukocyte adhesion molecule. *Proc Natl Acad Sci USA* 1987; 84: 9238–9242.
9. Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991; 349: 796–799.
10. Rothlein R, Dustin ML, Marlin SD, Springer TA. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *J Immunol* 1986; 137: 1270–1274.
11. Pober JS, Gimbrone MJ, Lapierre LA, et al. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumour necrosis factor, and immune interferon. *J Immunol* 1986; 137: 1893–1896.
12. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol* 1988; 107: 321–331.
13. Wellicome SM, Thornhill MH, Pitzalis C, et al. A monoclonal antibody that detects a novel antigen on endothelial cells that is induced by Tumour necrosis factor, IL-1 or Lipopolysaccharide. *J Immunol* 1990; 144: 2558–2565.
14. Griffiths CE, Voorhees JJ, Nickoloff BJ. Characterization of intercellular adhesion molecule-1 and HLA-DR expression in normal and inflamed skin: modulation by recombinant gamma interferon and tumour necrosis factor. *J Am Acad Dermatol* 1989; 20: 617–629.
15. Lewis RE, Buchsbaum M, Whitaker D, Murphey GF. Intercellular adhesion molecule expression in the evolving human cutaneous delayed hypersensitivity reaction. *J Invest Dermatol* 1989; 93: 672–677.
16. Munro JM, Pober JS, Cotran RS. Tumour necrosis factor and interferon-γ induce distinct patterns of endothelial cell activation and associated leukocyte accumulation in the skin of *Papio anubis*. *Am J Pathol* 1989; 135: 121–133.
17. Norris P, Poston RN, Thomas DS, Thornhill M, Hawk J, Haskard DO. The expression of Endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: A comparison of ultraviolet B erythema and delayed hypersensitivity. *J Invest Dermatol* 1991; 96: 763–770.
18. Crawford JM, Hopp B. Junctional epithelium expresses the intercellular adhesion molecule ICAM-1. *J Periodont Res* 1990; 25: 254–256.
19. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol* 1965; 36: 177–187.
20. Silness P, Loe H. Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. *Acta Odontol Scand* 1964; 22: 121–135.
21. Lobene RR, Weatherford T, Ross NM, Lamm RA, Menaker L. A modified gingival index for use in clinical trials. *Clin Prev Dent* 1986; 8: 3–6.
22. Loe H, Silness J. Periodontal disease in pregnancy I. Prevalence and severity. *Acta Odontol Scand* 1963; 21: 533–551.
23. Hsu S-M, Raine L. Protein A, avidin and biotin in immunohistochemistry. *J Histochem Cytochem* 1981; 29: 1349–1353.
24. Messadi DV, Pober JS, Fiers W, Gimbrone MA Jr, Murphy GF. Induction of an activation antigen on postcapillary venular endothelium in human skin organ culture. *J Immunol* 1987; 139: 1557–1562.
25. Munro JM, Pober JS, Cotran RS. Recruitment of neutrophils in the local endotoxin response: Association with de novo endothelial expression of endothelial leukocyte adhesion molecule-1. *Lab Invest* 1991; 64: 295–298.
26. Cotran RS, Gimbrone MA Jr, Bevilacqua MP, Mendrick DL, Pober JS. Induction and detection of a human endothelial activation antigen in vivo. *J Exp Med* 1986; 164: 661–666.
27. Bevilacqua MP, Stengelin S, Gimbrone MA Jr, Seed B. Endothelial leukocyte adhesion molecule 1: An inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* 1989; 243: 1160–1165.
28. Seymour GJ, Powell RN, Aitken JF. Experimental gingivitis in humans. A clinical and histologic investigation. *J Periodontol* 1983; 54: 522–528.
29. Seymour GJ, Powell RN, Cole KL, et al. Experimental gingivitis in humans – A histochemical and immunological characterisation of the lymphoid cell subpopulations. *J Periodont Res* 1983; 18: 375–385.
30. Smith CW, Martin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *J Clin Invest* 1989; 83: 2008–2017.
31. Dustin ML, Singer KH, Tuck DT, Springer TA. Adhesion of T-lymphoblasts to epidermal keratinocytes is regulated by interferon-γ and is mediated by intercellular adhesion molecule-1 (ICAM-1). *J Exp Med* 1988; 167: 1323–1340.
32. Barker JN, Allen MH, MacDonald DM. The effect of in vivo interferon-gamma on the distribution of LFA-1 and ICAM-1 in normal human skin. *J Invest Dermatol* 1989; 93: 439–442.
33. Krutmann J, Kock A, Schauer E, et al. Tumour necrosis factor b and ultraviolet radiation are potent regulators of human keratinocyte ICAM-1 expression. *J Invest Dermatol* 1990; 95: 127–131.
34. Anderson DC, Springer TA. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1 and p150,95 glycoproteins. *Ann Rev Med* 1987; 38: 175–194.
35. Page RC, Beatty P, Waldrop TC. Molecular basis for the functional abnormality in neutrophils from patients with generalized prepubertal periodontitis. *J Periodont Res* 1987; 22: 182–183.
36. Waldrop TC, Anderson DC, Hamilton WW, Schmalstieg FC, Jacobs RL. Periodontal manifestations of the hereditable Mac-1, LFA-1 deficiency syndrome: clinical, histopathologic and molecular characteristics. *J Periodontol* 1987; 58: 400–416.

Address:

Dr. D. F. Kinane
Department of Oral Medicine and Pathology
Glasgow Dental Hospital & School
378 Sauchiehall Street
Glasgow G2 3JZ
U.K.

J. Biol. Buccale, vol. 20. pp. XX à XX, 1992

LANGERHANS CELL DYNAMICS IN HUMAN GINGIVA DURING EXPERIMENTALLY INDUCED INFLAMMATION

N.A. MOUGHAL, E. ADONOGIANAKI, D.F. KINANE
Department of Oral Medicine and Pathology, Glasgow Dental Hospital & School, 378 Sauchiehall Street, Glasgow, G2 3JZ, UK

(Received November 19 1991 ; Accepted May 4 1992)

SUMMARY : The changes in Langerhans cell numbers within the gingiva during a 21 day experimental gingivitis episode were investigated immunohistochemically. Monoclonal antibodies to CD1a (specific for Langerhans cells and thymocytes) and HLA-DR (class II major histocompatibility antigens - (MHC)) were used to identify Langerhans cells within gingival biopsies taken on days 0, 7, 14 and 21. HLA-DR antibody stained dendritic cells within the oral epithelium which were morphologically identical to the CD1a$^+$ Langerhans cells. Class II MHC LC numbers rose and plateaued between day 7 and 14 then decreased to baseline by day 21. As plaque accumulated and initial inflammation developed there was an increase in the number of CD1a$^+$ Langerhans cells which peaked at day 7 and stayed high (day 14). As inflammation developed there was a statistically significant decrease in the number of CD1a$^+$ Langerhans cells by day 21 ($p = 0.028$). The initial increase, followed by a decrease in CD1a$^+$ Langerhans cells as inflammation developed, suggests that migration of Langerhans cells occurs within the gingival epithelium and this may represent an important early event in the gingival immune response to plaque.

KEY WORDS : Experimental gingivitis - Langerhans cells - HLA-DR - Inflammation - Oral epithelium

RESUME : Les modifications du nombre de cellules de Langerhans au niveau de la gencive ont été étudiées par immunohistochimie pendant une période de 21 jours de gingivite expérimentale. Les anticorps monoclonaux CD1a (spécifiques des cellules de Langerhans (LC) et des thymocytes) et HLA-DR (antigènes principaux d'histocompatibilité de classe II (MHC) ont été utilisés pour identifier les cellules de Langerhans dans les biopsies gingivales prélevées aux jours 0, 7, 14 et 21. L'anticorps HLA-DR a coloré des cellules dendritiques dans l'épithélium buccal qui sont identiques du point de vue morphologique aux cellules de Langerhans CD1a$^+$. Le nombre des cellules de Langerhans MHC classe II augmente et atteint un plateau entre le 7ème et le 14ème jour, puis revient aux valeurs de départ au jour 21. Au cours de l'accumulation de plaque et du développement initial de l'inflammation, on observe une augmentation du nombre des cellules de Langerhans CD1A+ qui atteint un pic au jour 7, puis reste élevé au jour 14. Avec le développement de l'inflammation, on note une diminution statistiquement significative du nombre des LC CD1a$^+$ au jour 21 ($p = 0.028$). L'augmentation initiale suivie d'une diminution des LC CD1a$^+$ au cours du développement de l'inflammation suggèrent qu'une migration des LC a lieu dans l'épithélium gingival et représente un évènement précoce important de la réponse immunitaire gingivale à la plaque dentaire.

MOTS CLES : Gingivite expérimentale - Cellules de Langerhans - HLA-DR - Inflammation - Epithélium buccal

INTRODUCTION

Langerhans cells (LC) are dendritic, suprabasal cells found in most stratified squamous epithelia including epithelia of the oral mucosa (Daniels, 1984). They are of bone marrow origin (Katz et al., 1979) and share many characteristics in common with the macrophage (Thorbecke et al., 1980). In addition, LCs express Fc IgG receptors and C3 receptors (Stingl et al., 1977) as well as CD1a and Class II major histocompatibility (MHC) antigens (Klareskog et al., 1977 ; Rowden et al., 1977) and are capable of antigen presentation (Silberberg-Sinakin et al., 1980 ; Walsh et al., 1988 ; Cruchley et al., 1989).

Hitzig et al., (1989) reported a cross-sectional study where gingival tissue was categorised according to clinical indices. These authors suggested LC numbers increased during early stages of inflammation and decreased in severe inflammation. These cross-sectional findings on chronic gingivitis, are in contrast to longitudinal experimental gingivitis studies where LC numbers were reported to increase steadily with gingival inflammation (Newcomb et al., 1982 ; DiFranco et al., 1985 ; Newcomb and Powell, 1986a, 1986b).

The aim of the present study was to examine changes in LC numbers within human gingival tissues during experimentally induced gingivitis. The monoclonal antibody to CD1a (cluster of differentiation (CD) antigen 1a) (Fithian et al., 1981 ; Harrist et al., 1983 ; DiFranco et al., 1985) was used in the immunohistochemical analysis of LC numbers in the biopsy material. Langerhans cells have been shown to be HLA-DR+ (Klareskog et al., 1977) and related to this, are capable of antigen presentation (Stingl et al., 1978), therefore HLA-DR positivity of LCs was also assessed.

METHODS

Biopsy material was obtained using the experimental gingivitis model of Löe, Theilade and Jensen (1965). Ethical approval was granted by the local ethical committee. Five dental students (4 male, 1 female) who consented to participate in the study, received oral hygiene instructions and frequent prophylaxis until maximal clinical gingival health was obtained. All subjects were then instructed to cease all oral hygiene measures and plaque was allowed to accumulate undisturbed for a period of 21 days. The clinical indices were recorded (every 7 days) at each biopsy site, using the Plaque Index (PI) of Silness and Löe (1964) and the Modified Gingival Index (MGI) of Lobene et al. (1986). The MGI is a modification of the Löe and Silness (1963) Gingival index, it omits the bleeding-upon-probing component of the index and permits a non-invasive evaluation of the early visible changes in the extent and severity of gingivitis. One examiner was used throughout the experiment to record the clinical indices (E.A.).

Gingival biopsies (2mm x 2mm) were obtained surgically on day 0 of the experimental gingivitis phase, from the mid-buccal aspect of the lower right first permanent molar. The remaining first permanent molars were then biopsied in rotation (day 7, 14 and 21). In total four biopsies, one at each time point, were taken from each individual. All tissue biopsies were embedded in Tissue Tek and snap frozen, 8um thick sections were cut and stained using the ABC method of Hsu, Raine and Fanger (1981). Frozen cryostat sections of the specimen were air dried and fixed in cold acetone. Following fixation, the sections were blocked with horse serum before the addition of the primary monoclonal antibodies. The sections were reacted for 1 hour at room temperature (RT) with a 1 : 50 dilution of the mouse monoclonal antibodies OKT6 (anti-CD1a) (Ortho Diagnostics, Buckinghamshire, UK) or OKIa1 (anti-HLA-DR) (Ortho Diagnostics, Buckinghamshire, UK). The primary monoclonal antibodies were then labelled with biotinylated anti-mouse immunoglobulin. Before the addition of avidin-biotinylated peroxidase complex (Vector Laboratories, Peterborough, U.K.) the sections were deperoxidised (0.03 % hydrogen peroxide in methanol) to remove endogenous peroxidase. Between each incubation the slides were washed with phosphate buffered saline (pH 7.4). The reaction was developed using the substrate diaminobenzidine tetrahydrochloride and to enhance staining, the sections were then incubated in 0.5 % copper sulphate solution. The slides were finally counterstained with haematoxylin, dehydrated and mounted. One section from each biopsy was also stained with haematoxylin and eosin. Monoclonal antibody specificities were confirmed using human tonsil and human skin as control tissue and appropriate negative control sections treated with non-specific primary mouse monoclonal antibodies (CD21 and CD22, both IgG1).

Only sections with intact gingival oral epithelium (OE) were used for LC quantification. LCs were identified as dendritic nucleated, CD1a+ or HLA-DR+ cells, within the full thickness of the gingival OE. Ten randomly chosen high power fields from biopsies (minimum = 3 sections) from each individual at each time point were studied and LCs were counted "blind" at x 400 magnification. The area of sectioned gingival OE was measured from each section using computer assisted image analysis (Video Vector Dynamics, Glasgow, U.K.) and the mean number of positive cells per biopsy calculated and expressed as LCs per high power field (0.018 mm2). Serial sections from day 0 and day 7 biopsies were prepared to permit calculation of the percentage of HLA-DR+ CD1a+ LCs.

Repeated measures analysis of variance (SPSS PC) were used to determine if 'day' effects were significant for both CD1a+ and HLA-DR+ expression on LCs during the experimental gingivitis experiment. Paired t-tests were then used to compare between days, and a multiple comparison correction employed. Lack of a biopsy for subject A on day 0 necessitated omitting this subject from the repeated measures analysis.

RESULTS

Following the withdrawal of oral hygiene procedures the clinical indices of the biopsy sites rose and peaked by day 21, with the rise in PI just preceding the rise in the MGI (Table I). At day 0 in healthy gingiva, CD1a+ LCs were seen within the oral and sulcular epithelia. Generally they were dendritic and were found within the stratum spinosum of the gingival OE, with only a few cells present within the sulcular epithelium. Junctional epithelium was often incomplete and variable and the term sulcular epithelium was used to include both junctional and oral sulcular epithelium. As plaque accumulated there was a shift of LCs from the stratum spinosum to the stratum granulosum (day 0 to day 7) and by day 14 the CD1a+ LCs were more basally positioned (Fig. 1). HLA-DR+ dendritic cells were found within the gingival OE and were morphologically identical to the CD1a+ LCs (Fig. 2) and this evidence together with the serial section data, strongly suggests that the two monoclonal antibodies are detecting the same cell type within the gingival OE. HLA-DR antigens were detected on 52.5 % (day 0) and 82.1 % (day 7) of

CD1a+ LCs (calculated from serial sections). Keratinocytes only faintly stained for HLA-DR and combined with their diffuse expression permitted clear distinction from LCs to be made.

As the clinical indices increased, the density of CD1a+ LCs within the gingival OE increased during the first seven days of oral hygiene abstention. Following this initial increase, CD1a+ LC numbers decreased only slightly with further plaque accumulation and development of gingival inflammation (day 7 to day 14). However, as inflammation developed (day 14 to day 21), the numbers of CD1a+ LCs detected within the gingival OE decreased back to baseline levels by day 21 (Table I). The number of HLA-DR+ LCs however increased and plateaued between day 7 and day 14, then decreased to baseline levels by day 21 (Table I).

Repeated measures analysis of variance (MANOVA) demonstrated significant changes in the numbers of CD1a+ LCs with time and further analysis (paired t-tests) demonstrated that the decrease in CD1a+ LCs which occurred between day 7 and 21 and between day 14 and 21 were statistically significant ($p = 0.003$ and $p = 0.028$ respectively). However, no statistically significance was demonstrated when MANOVA was used to analyse HLA-DR+ LCs changes. The statistical levels for these analyses must be reduced as they were part of multiple comparisons ($n = 6$) and a therefore a statistically significant threshold of 0.02 should be used.

DISCUSSION

Conflicting data exists on the dynamics of LC numbers during gingival inflammation. Many workers have suggested that as inflammation develops there is an increase in the number of LCs present within gingival oral epithelium (Newcomb et al., 1982 ; DiFranco et al., 1985 ; Newcomb and Powell, 1986a, 1986b). However, a recent cross-sectional study by Hitzig et al., (1989) where tissue was categorised according to clinical indices, suggested that during initial inflammation there was an increase in LC numbers, and in severe inflammation this decreased, contradicting previous studies. This

Table I. Clinical parameters and Langerhans cell numbers (CD1A+ and HLA-DR+) during the 21 days of experimental gingivitis. Means with standard deviations in parenthesis are shown for CD1A and HLA-DR positive Langerhans cells in 10 high power fields (0.018 mm2)

DAY	SUBJECT	PI	MGI	CD1A+LCS per 0.018 mm^2	HLA-DR+ LCs per 0.018 mm^2
0	A	0	0	Not done	Not done
	B	1	0	4.2 (1.3)	0.8 (0.6)
	C	0	0	6.2 (1.3)	2.3 (1.1)
	D	0	0	3.7 (1.1)	0.3 (0.5)
	E	0	0	4.9 (1.9)	0.5 (0.7)
	mean			4.8 (1.1)	1.0 (0.8)
7	A	1	0	5.8 (1.1)	2.3 (0.7)
	B	1	1	7.0 (2.3)	1.3 (0.9)
	C	2	1	6.3 (2.0)	1.4 (0.7)
	D	2	1	5.8 (1.8)	2.0 (1.1)
	E	2	2	4.6 (1.1)	3.4 (0.8)
	mean			5.9 (0.8)	2.1 (0.8)
14	A	2	2	5.2 (1.5)	2.9 (1.0)
	B	2	2	5.1 (1.8)	0.6 (1.0)
	C	2	2	5.8 (1.3)	0.8 (0.6)
	D	2	2	5.3 (1.2)	3.8 (1.8)
	E	2	2	5.3 (1.2)	2.2 (1.5)
	mean			5.3 (0.3)	1.9 (1.8)
21	A	2	2	5.1 (1.6)	0.0 (0.0)
	B	3	3	4.0 (1.9)	1.1(0.7)
	C	2	2	4.3 (1.1)	1.0 (0.6)
	D	2	2	2.8 (1.5)	1.8 (0.6)
	E	2	2	1.8 (1.3)	1.9 (1.0)
	mean			3.6 (1.3)	1.2 (0.8)

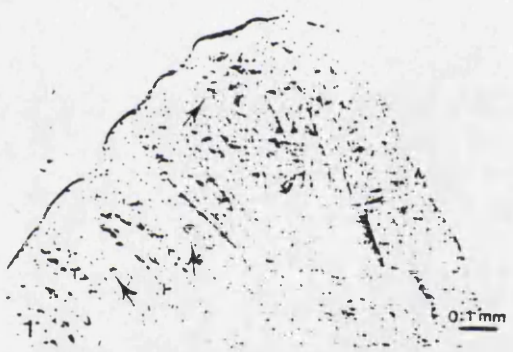

Fig. 1. Day 14 biopsy with CD1a+ Langerhans cells within the oral epithelium (solid arrows) (Immunoperoxidase) (x 78.75).

Fig. 2. Day 7 biopsy : A HLA-DR+ Langerhans cells within the oral epithelium (solid arrow) (Immunoperoxidase) (X 500).

work provides an insight into the changes in LC numbers within the gingival OE at different stages of inflammation. Results from the present study support the view of Hitzig et al., (1989) that there is initially an increase in LC numbers followed by a decrease as inflammation develops.

DiFranco et al., (1985), however compared treated and untreated lesions within the same individual using OKT6, S-100 and HLA-DR markers. These workers found that the numbers of LCs present in the inflamed tissue were significantly increased, and that the best marker for LCs was OKT6 which gave a maximum number of LCs in the gingival epithelium of 9.8 LC/mm^2. This is extremely low when compared to both the maximum number of LCs reported in the present study (389 LC/mm^2) and to the study of Hitzig et al.(1989) (117 LC/mm^2), suggesting that the absolute numbers of LCs found within these diseased subjects was severely depressed. Interestingly, Hitzig et al., (1989) used thinner sections 6µm, than the 8µm of the present study, and this plus a more restrictive criteria for LC determination may account for the lower numbers seen. Newcomb et al., (1982) employed Adenosine Triphosphatase to compare LC numbers in gingival tissue obtained after 0, 8 and 21 days of plaque accumulation in 4 volunteers. These workers found a statistically significant increase in the number of LCs present as plaque accumulated (day 0 to day 8). However, with the development of gingival inflammation (day 8 to day 21), there was no difference in the number of LCs present. The present study reports a peak in the number of LCs present after 7 days of plaque accumulation, and a statistically significant decrease in CD1a$^+$ LCs seen between days 7 and 21 and days 14 and day 21 (Table I) ($p = 0.003$ and $p = 0.028$ respectively) (MANOVA). Newcomb and Powell (1986b) have more recently demonstrated that adenosine triphosphatase is not as specific for LCs as the CD1a monoclonal antibody.

The above studies examined LC numbers in different individuals ; diseased compared with healthy individuals or at different time points, sites within the same diseased individual. Closer evaluation of the data from these studies suggests that the numbers of LCs detected within these subjects are severely decreased in comparison to the present study.

The number of Class II MHC positive (HLA-DR$^+$) LCs plateaued between day 7 and 14 and returned to baseline levels by day 21. As the number of CD1a$^+$ LCs remained relatively constant between day 7 and day 14, this might be explained if the CD1a$^+$ LCs present on day 0 consisted of some HLA-DR$^-$ cells which become class II$^+$ cells as inflammation developed (Walsh et al., 1988). The HLA-DR specific monoclonal antibody could also be detecting macrophages. Further histochemical analysis however, using the macrophage specific OKM1 monoclonal antibody (Ortho), suggested that macrophages were too infrequent within the epithelium to appreciably effect the numbers of HLA-DR$^+$ cells. The percentage of CD1a$^+$ LCs which also expressed HLA-DR antigen varied from 52.5 to 82.1 % (calculated from day 0 and day 7 serial sections repectively), whereas the CD1a and HLA-DR positive LCs given in Table I, albeit from non-serial sections indicate a range in this study between 0 to 75 %. This lower percentage and wide variation in the HLA-DR expression of CD1a+ LCs derived from non-serial sections indicates the advantages of serial sections when enumerating two antigens on one cell type.

The inflammatory reaction produced in experimental gingivitis results in various cells releasing cytokines and lymphokines (Charon et al., 1982 ; Mergenhagen 1984 ; O'Neill and Woodson, 1986 ; Kinane et al., 1992). The differential effects of cytokines on LCs surface antigen expression may modulate the subpopulation of LCs present (Walsh et al., 1988 ; Ishii et al., 1990). Walsh et al., (1990) have demonstrated that Class II MHC positive (HLA-DR$^+$) LCs are required for effective antigen presentation and that this expression is important for gingival LCs to initiate a T lymphocyte response to antigen. It is unclear of what significance the LC number changes reported here are, but a flux of LCs into and out of the gingival epithelium may be occurring analogous to the changes seen in the number of LCs present at the reaction site during contact hypersensitivity (Silberberg-Sinakin et al., 1980).

In conclusion, changes in LC numbers occurs within the first weeks of gingival inflammation. This may reflect movement in and out of gingival epithelium and suggests that these cells are active in antigen presentation even at this early stage of inflammation. Migration of gingival LCs into the lymphoid system would result in stimulation of lymphocytes and initiation of an immune response. This flux of LCs may represent an important early "immune triggering" event in the gingival immune response to plaque.

ACKNOWLEDGEMENTS : The authors would like to thank Professor D.G. Mac Donald for his assistance with the stereological procedures and also Professor P.C. Wilkinson and Dr M.H. Thornhill for their helpful comments. Support from the Medical Research Funds Committee of the University of Glasgow is gratefully acknowledged.

REFERENCES

Charon J.A., Luger T.A., Mergenhagen S.E. and Oppenheim J.J. - Increased thymocyte-activating factor in human gingival fluid during gingival inflammation. *Infect. Immun.*, 38 : 1190-1195, 1982.

Cruchley A.T., Williams D.M., Farthing P.M., Lesch C.A. and Squire C.A. - Regional variation in Langerhans cell distribution and density in normal human oral mucosa determined using monoclonal antibodies against CD1, HLADR, HLADQ and HLADP. *J. Oral Pathol. Med.*, 18 : 510-516, 1989.

Daniels T.E. - Human mucosal Langerhans cells : postmortem identification of regional variations in oral mucosa. *J. Invest. Dermatol.*, 82 : 21-24, 1984.

DiFranco C.F., Toto P.D., Rowden G., Gargiulo A.W., Keene J.J.Jr. and Connelly E. - Identification of Langerhans cells in human gingival epithelium. *J. Periodontol.*, 56 : 48-54, 1985.

Fithian E., Kung P., Goldstein G., Rubenfeld M., Fenoglio C. and Edelson R. - Reactivity of Langerhans cells with hybridoma antibody. *Proc. Natl. Acad. Sci. U. S. A.* 78 : 2541-2544, 1981.

Harrist T.J., Muhlbauer J.E., Murphy G.F., Mihm M.C. Jr. and Bhan A.K. - T6 is superior to Ia (HLA-DR) as a marker for Langerhans cells and indeterminate cells in normal epidermis: A monoclonal antibody study. *J. Invest. Dermatol.*, 80 : 100-103, 1983.

Hitzig C., Monteil R.A., Charbit Y. and Teboul M. - Quantification of T6+ and HLA/DR+ Langerhans cells in normal and inflamed human gingiva. *J. Biol. Buccale*, 17 : 103-108, 1989.

Hsu S.M., Raine L. and Fanger H. - Use of Avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques : a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.*, 29 : 577-580, 1981.

Ishii T., Walsh L.J., Seymour G.J. and Powell R.N. - Modulation of Langerhans cell surface antigen expression by recombinant cytokines. *J. Oral Pathol. Med.*, 19 : 355-359, 1990.

Katz S.I., Tamaki K. and Sachs D.H. - Epidermal Langerhans cells are derived from cells originating in bone marrow. *Nature*, 282 : 324-326, 1979.

Kinane D.F., Winstanley F.P., Adonogianaki E. and Moughal N.A. - Bioassay of interleukin 1 (IL-I) in human gingival crevicular fluid during experimental gingivitis. *Arch. oral Biol.*, 37 : 153-156, 1992.

Klareskog L., Tjernlund U.M., Forsum U. and Peterson P.A. - Epidermal Langerhans cells express Ia antigens. *Nature*, 268 : 248-250, 1977.

Lobene R.R., Weatherford T., Ross N.M., Lamm R.A. and Menaker L. - A modified gingival index for use in clinical trials. *Clin. Prev. Dent.*, 8 : 3-6, 1986.

Löe H., Theilade E. and Jensen S.B. - Experimental gingivitis in man. *J. Periodontol.*, 36 : 177-187, 1965.

Löe H. and Silness J. - Periodontal disease in pregnancy I. Prevalence and severity. *Acta. Odontol. Scand.*, 21 : 533-551, 1963.

Mergenhagen S.E. - Thymocyte activating factor(s) in human gingival fluids. *J. Dent. Res.*, 63 : 461-464, 1984.

Newcomb G.M. and Powell R.N. - The ultrastructure of human gingival Langerhans cells in health and disease. *Arch. oral Biol.*, 31 : 727-734, 1986a.

Newcomb G.M. and Powell R.N. - Human gingival Langerhans cells in health and disease. *J. Periodont. Res.*, 21 : 640-652, 1986b.

Newcomb G.M., Seymour G.J. and Powell R.N. - Association between plaque accumulation and Langerhans cell numbers in the oral epithelium of attached gingiva. *J. Clin. Periodontol.*, 9 : 297-304, 1982.

O'Neill P.A. and Woodson D.L. - Lymphokine production by human gingival lymphocytes. *J. Periodont. Res.*, 21 : 338-350, 1986.

Rowden G., Lewis M.G. and Sullivan A.K. - Ia antigen expression on human epidermal Langerhans cells. *Nature*, 268 : 247-248, 1977.

Silberberg-Sinakin I., Gigli I., Baer R.L. and Thorbecke G.J. - Langerhans cells : Role in contact hypersensitivity and relationship to lymphoid dendritic cells and to macrophages. *Immunol. Rev.*, 53 : 203-232, 1980.

Silness P. and Loe H. - Periodontal disease in pregnancy II. Correlation between oral hygiene and periodontal condition. *Acta Odontol. Scand.*, 22 : 121-135, 1964.

Stingl G., Katz S.I., Clement L. and Shevach E.M. - Immunological function of Ia-bearing epidermal Langerhans cells. *J. Immunol.*, 121 : 2005-2013, 1978.

Stingl G., Wolff-Schreiner E.C., Pichler W.J., Gschnait F. and Knapp W. - Epidermal Langerhans cells bear Fc and C3 receptors. *Nature*, 268 : 245-246, 1977.

Thorbecke G.J., Silberberg-Sinakin I. and Flotte T.J. - Langerhans cells as macrophages in skin and lymphoid organs. *J. Invest. Dermatol.*, 75 : 32-43, 1980.

Walsh L.J., Seymour G.J. and Powell R.N. - The regulation of Langerhans cell T6, DR and DQ antigen expression: an hypothesis. *J. Oral Pathol.*, 17 : 43-46, 1988.

Walsh L.J., Seymour G.J. and Powell R.N. - Human gingival Langerhans cells stimulate allogeneic lymphocytes : Requirements for MHC Class II antigens. *J. Periodontol.*, 61 : 328-333, 1990.

Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases

E. Adonogianaki, N. A. Moughal and D. F. Kinane

Periodontal Unit, Department of Oral Medicine & Pathology, Glasgow Dental Hospital and School, 378 Sauchiehall Street, Glasgow, G2, 3JZ, UK

Adonogianaki E, Moughal NA and Kinane DF: Lactoferrin in the gingival crevice as a marker of polymorphonuclear leucocytes in periodontal diseases. J Clin Periodontol 1992; 19: 000–000.

Abstract. This study examined lactoferrin (LF) levels in gingival crevicular fluid (GCF) and set out to test the hypothesis that LF could act as a marker of crevicular polymorphonuclear leucocytes (PMN). Therefore, 2 experiments were conducted: (a) to quantify total LF (ng/30 s sample) in GCF; (b) to correlate LF levels (ng/μl) and PMN numbers (PMNs/μl) in gingival crevicular washings (GCW). GCF was collected from 71 sites in a total of 22 patients. These sites were classified on the basis of clinical indices of gingivitis (GI) and pocket depth (PD) into three clinical groups: 'healthy', 'gingivitis' and 'periodontitis'. GCWs were obtained from an additional 63 sites in 21 patients. LF in GCF and GCWs was assayed by a sandwich ELISA. Total leucocyte and differential counts were performed on the GCWs. GCF LF (ng/30 s) correlated positively with GI ($r=0.418$, $p<0.001$), PD ($r=0.415$, $p<0.001$) and GCF volume ($r=0.624$, $p<0.001$). Gingivitis ($n=21$) and periodontitis sites ($n=24$) demonstrated significantly higher ($p<0.05$) total GCF LF than healthy ($n=26$) sites. In GCWs LF (ng/μl) showed stronger correlations with clinical indices (GI: $r=0.452$, PD: $r=0.513$, $p<0.001$) than did PMN numbers (PMNs/μl) (GI: $r=0.279$, PD: $r=0.388$, $p<0.05$). LF correlated strongly with PMNs in GCWs ($r=0.531$, $p=0.001$) and provides a simple and effective marker of crevicular PMN numbers.

Key words: gingival crevicular fluid; gingival crevicular washings; lactoferrin; polymorphonuclear leucocytes.

Accepted for publication 4 December 1991

The polymorphonuclear leucocyte (PMN) is the predominant leucocyte within the gingival crevice in both health and disease (Attstrom & Egelberg 1970, Kowashi et al. 1980, Thurre et al. 1984). Qualitative and quantitative PMN deficiencies result in gross periodontal destruction (Wilton et al. 1988, Kinane & Davies, 1990). Although the PMN has a protective role, the interplay between microbial plaque components, complement, immune complexes and PMNs, initiates the release of PMN intracellular contents during inflammation (Henson 1971a, Henson 1971b, Taichman et al. 1977) which may damage the neighbouring tissues (Wilton 1986). The PMN therefore appears to have both a protective and potentially destructive role in the pathogenesis of periodontal disease. Granulocytes dominate periodontal inflammation (Gillett et al. 1990) and it has been suggested that PMN numbers in the crevice could be a useful marker of the inflammatory status of specific sites (Fine & Mandel 1986). PMN numbers in gingival washings have been shown to increase with the development of experimental gingivitis (Kowashi et al. 1980, Thurre et al. 1984) and higher PMN numbers have been obtained at periodontitis when compared to gingivitis sites (Thurre et al. 1984).

Sampling gingival crevicular fluid (GCF) provides a non-invasive means of quantifying site specific biochemical parameters which could help identify the periodontal status of different sites. Numerous PMN constituents, mainly enzymes, have been studied in GCF but most of these are not exclusive to the PMN (Fine & Mandel 1986, Curtis et al. 1989). Lactoferrin (LF) is an iron binding protein present specifically and in abundance in PMN secondary granules (Spitznagel et al. 1974) but not in other leucocytes (Bennet & Kokocinski, 1978) and only in trace amounts in serum (Hetherington et al. 1983). PMN secondary granules are more numerous than primary granules (Falloon & Gallin, 1986) and their contents are released earlier and more readily during in vitro PMN stimulation (Bentwood & Henson, 1980). Moreover, PMN secondary granules have been shown to be preferentially released, over primary granules, during both adherence in vitro and migration through epithelium in vivo (Wright & Gallin, 1979). Wilton (1986) summarised the stimuli for PMN degranulation in the crevice and concluded that PMN secondary granule release is more likely in vivo. It has been suggested therefore that LF in GCF could perhaps prove a reliable marker of PMN emigration and/or activation in the crevice (Fine & Mandel 1986, Curtis et al. 1989).

Crevicular LF may act as an antimicrobial agent by creating an iron

limiting environment due to its high affinity for iron, although it may also exert a direct bactericidal effect independent of iron deprivation (Arnold et al. 1982). In addition, LF facilitates phagocytosis of plaque bacteria by reducing their hydrophobicity and preventing their adherence (Wilton 1986). LF may also be implicated in the inflammatory response by enhancing PMN adhesiveness and chemotaxis (Oseas et al. 1981, Falloon & Gallin 1986), by modulating production of interleukin-1 (Zucali et al. 1989) and prostaglandins (Pelus et al. 1979, Bartal et al. 1987) and by scavenging iron which could catalyse free hydroxyl radical formation (Britigan et al. 1989). Although promising as a periodontal disease activity marker and/or modulator, LF in GCF has only been reported in one study to date by Friedman et al. (1983), who found higher LF concentration in pooled GCF from gingivitis, periodontitis and localised periodontitis patients when compared to healthy controls.

The present study was undertaken firstly, to assess LF levels in discrete GCF samples and to determine their correlation with the clinical indices of the sites sampled, and secondly, to test the hypothesis that LF could act as a marker of PMN emigration into the crevice. In order to address both issues, experiments were conducted: (a) to quantify LF in GCF; (b) to compare LF levels and PMN numbers in gingival crevicular washings (GCW).

Material and Methods

Clinical criteria

43 individuals attending the Glasgow Dental Hospital were selected to participate in this study. These individuals were all Caucasian, had no history of systemic disease and had not received any antibiotics for the past 3 months. 32 of these individuals (APD category) suffered from advanced periodontal disease as determined by clinical and radiographic examination. The additional ten subjects (H category) could be classified as periodontally healthy (no attachment loss, pocket depths ≤ 3 mm and no significant gingival inflammation).

GCF samples were collected from 22 of the above individuals (age range 22 to 51 years; 10 male, 12 female; 16 APD, 6 H) and GCWs from the remaining twenty one individuals (age range 20 to 52 years; 7 male, 14 female; 16 APD, 5 H). A maximum of 4 sites were sampled per subject. The evaluation of gingival inflammation of the sites sampled were performed by the modified gingival index (GI) (Lobene et al. 1986) and the pocket depth (PD) was assessed (after GCF or GCW sampling) to the nearest mm using a Williams periodontal probe. Diseased sites ($GI \geq 2$, $PD \geq 3$ mm) were obtained from the periodontitis patients whereas healthy sites ($GI \leq 1$, $PD \leq 2$ mm) were obtained mainly from the periodontally healthy subjects and in some cases from the periodontitis patients.

PMN numbers were assessed only in GCWs whereas LF levels were quantified in both GCF and GCW samples. For the establishment of the range of LF levels in GCF according to the clinical presentation of the sites sampled, the latter were allocated into three clinical groups: 'healthy', 'gingivitis' and 'periodontitis'. Sites with gingival scoring of 0 or 1 and pocket depth not exceeding 2 mm were categorised as 'healthy'. GI between 2 and 4, and pocket depth less than or equal to 3 mm were the criteria for the 'gingivitis' sites, whereas sites with pocket depth exceeding 3 mm were designated 'periodontitis' sites.

Gingival crevicular fluid sampling and processing

Whatman grade 4 paper strips (2 × 13 mm) were used for GCF collection (Griffiths et al., 1988). The individual crevicular site was gently air-dried and any visible supragingival plaque was removed. The area was carefully isolated with cotton rolls and saliva ejector to avoid saliva contamination of the samples. The paper strip was introduced into the crevice until mild resistance was felt and left for 30 s. Care was taken in order to avoid mechanical injury of the tissues. After GCF collection, the paper strip was transferred to the chairside-located Periotron (Periotron 6000, Harco Electronics, Winnipeg, Canada) for the assessment of fluid volume. The jaws of the Periotron were wiped with pure methanol between sequential readings. The strips were then placed in individual sterile microcentrifuge tubes and stored at −30°C until further processing. Subsequently, the strips were eluted into 1 ml of phosphate buffered saline containing 0.05% Tween 20 (PBST) and 0.1% bovine serum albumin (BSA) for 1 h using a rotating mixer. The strips were then discarded and the eluate aliquoted and stored at −70°C until used for the quantitation of lactoferrin. Prior to assaying the sample was diluted further in the same buffer.

Gingival crevicular washings

GCWs were obtained using a modification of the Skapski & Lehner method (1976). The teeth were isolated with saliva ejector and cotton rolls and any visible supragingival plaque was removed. Aliquots of 20 μl of Phosphate Buffered Saline (PBS) were ejected and reaspirated in the crevice 3 × using a 20 μl micropipette, fitted with a flat ended pipette tip (Labsystems, UK), resting interdentally on the surface of the tooth. The gingival washings were then stored in individual microcentrifuge tubes at 4°C. 1 μl aliquot of the washing, obtained using a Hamilton Microsyringe, was eluted in 1 ml of PBST (0.1% BSA) and the eluate was stored separately at −70°C for subsequent quantitation of GCW LF.

Total and differential white cell counts

Within 30 min of GCW collection a total cell count was performed on 5 μl of the sample. A differential count of 200–300 cells into polymorphonuclear and mononuclear cells was carried out after cytocentrifuging the rest of the sample at 70 g (Shandon Scientific Limited, UK) and staining the cells by the Leishman method. The number of PMN/μl GCW was finally calculated using the formula: total cell count × % PMNs/100.

LF quantification

LF quantitation was performed on the eluate of GCWs and GCF. A modification of the technique described by Hetherington et al. (1983) was applied. Goat anti-LF (Nordic) was coated on the polystyrene microplate (Immulon IV, Dynatech laboratories). The eluate was added and any LF present was captured by the immobilized antibody. Incubation with the second anti-LF (rabbit) (Nordic) was followed by addition of peroxidase conjugated anti-rabbit IgG (goat) (ICN). Each plate included positive and negative controls and serial dilutions of purified LF (Calbiochem) to permit construction of a standard curve from which sample LF quantities could be estimated. Visualisation was achieved by incubation with the sub-

Table 1. Spearman rank correlation coefficients (r) between clinical indices and GCF LF levels of $n=71$ sites sampled; probability levels (p) are shown in parenthesis

Parameter	LF (ng/30 s sample)
GI	$r=0.418$ ($p<0.001$)
PD	$r=0.415$ ($p<0.001$)
GCF (μl)	$r=0.624$ ($p<0.001$)

strate (o-phenylenediamine) and stopping the reaction with H_2SO_4. The plate was read at 490 nm.

LF levels in GCF samples were expressed as absolute amounts in ng per 30 s sample (ng/30 s sample). In GCWs LF levels were expressed as ng/μl GCW.

Statistical analysis

LF levels in GCF samples were skewed and were transformed to the base 10 ($\log_{10}$) in order to normalise their distribution and to obtain representative means of LF levels in the three clinical groups, 'healthy', 'gingivitis' and 'periodontitis'. For the same reason the 95% confidence intervals were preferred rather than the standard error (SE) or standard deviation (SD). Significant differences of GCF LF mean levels among the clinical groups were determined by two sample t-test on the $\log_{10}$ transformed data.

Associations between LF levels (GCF and GCWs) or PMN numbers (GCWs) and the clinical indices (GI, PD and GCF volume) were determined using the non-parametric Spearman rank correlation coefficient. The association between LF levels (ng/μl GCW) and PMN numbers (PMNs/μl GCW) in GCWs was determined by the Pearson correlation coefficient on the $\log_{10}$ transformed data. Data were analyzed using the 'Minitab' statistical package on the IBM PC computer.

Results

Gingival crevicular fluid

Seventy one sites were sampled in total for GCF. Associations between LF levels in GCF (ng/30 s sample) and the clinical indices were determined, and the Spearman rank correlation coefficients and their significance levels are shown in Table 1. Absolute amounts of GCF LF demonstrated positive and significant correlations with GI, PD and GCF volume.

Table 2 shows the sites allocated to the three clinical groups, 'healthy' ($n=26$), 'gingivitis' ($n=21$) and 'periodontitis' ($n=24$). Higher GCF LF levels (ng/30 s sample) from gingivitis ($p=0.012$) and periodontitis ($p=0.0023$) sites were obtained when compared to healthy sites (Table 2). No significant difference in LF levels between gingivitis and periodontitis sites was demonstrated ($p>0.05$) (Table 2).

Gingival crevicular washings

A total of 63 sites were sampled for GCWs. The mean GI and PD of the sites sampled were 1.87 (SD:1.28) and 3.19 mm (SD:2.2) respectively. Both LF levels (ng/μl GCW) and PMN numbers (PMNs/μl GCW) were determined in each sample. The relationship between GCW LF levels and PMN numbers was examined, the Pearson correlation coefficient was determined ($r=0.531$, $p<0.001$, $n=63$) and the least squares method was used in order to plot the best fitting line (Fig. 1). The association between both GCW LF levels and GCW PMN numbers and the clinical indices was also assessed (Table 3). GCW LF levels and PMN numbers correlated positively and significantly with both GI and PD, but LF demonstrated consistently higher correlation coefficients than PMNs with both clinical indices (Table 3).

Discussion

Recently, a method has been described for the approximate determination of PMN numbers in discrete GCF samples, collected with paper strips (Cimasoni & Giannopoulou 1988). This technique would appear to permit assessment of LF levels and PMN numbers from the same GCF sample. However, in a pilot study performed in our clinics and laboratories on a limited number of GCF samples, no PMNs could be recovered from the strips, either when vortexing (Cimasoni & Giannopoulou 1988) or centrifuging the sample (Ebersole et al. 1984) (data not shown). Thus, we resorted to the semi-quantitative method of GCWs for the determination of PMNs in the crevice and obtaining their relationship with GCW LF. Moreover, each site was sampled only once, either for GCF or GCW, as it was felt disruption by either sampling method would have affected the results obtained by the subsequent sampling technique. Therefore, 2 different experiments, on GCF and GCWs from discrete sites, were conducted so as to address the two central issues: (a) what levels of LF are present in GCF and how do they relate to the clinical condition of the sites sampled? and (b) can crevicular LF be used as a marker for PMNs in the area?

We chose to report GCF LF levels as absolute amounts in ng/30 s sample. The rationale for expressing PMN constituents in GCF as absolute amounts with a standardised collection time has been discussed in detail by several investigators (Lamster et al. 1986, Smith & Geegan 1991). In contrast, LF levels and PMNs in GCWs were expressed as concentrations (ng/μl GCW, PMNs/μl

Table 2. Clinical indices and LF levels at healthy gingivitis and periodontitis sites; the mean (±SE) clinical indices and GCF volume are shown; the mean LF levels, expressed as ng/30 s sample, are given; figures in parenthesis indicate the 95% confidence intervals

	Site		
Parameter	Healthy ($n=26$)	Gingivitis ($n=21$)	Periodontitis ($n=24$)
GI	0.38±0.10	2.57±0.11	2.46±0.17
PD	1.54±0.10	2.71±0.10	5.04±0.29
GCF (μl)	0.09±0.02	0.32±0.06	0.35±0.06
LF (ng/30 s) sample	95 (68–132)	181 (123–266)[a]	217 (144–328)[b]

[a] Significantly different from healthy sites $p<0.02$.
[b] Significantly different from healthy sites $p<0.003$.

Table 3. Spearman rank correlation coefficients (r) between clinical indices and GCW LF levels and PMN numbers of $n=63$ GCWs obtained; probability levels (p) are shown in parenthesis

Parameter	LF (ng/μl GCW)	PMNs (PMNs/μl GCW)
GI	$r=0.452$ ($p<0.001$)	$r=0.279$ ($p<0.030$)
PD	$r=0.513$ ($p<0.001$)	$r=0.388$ ($p<0.003$)

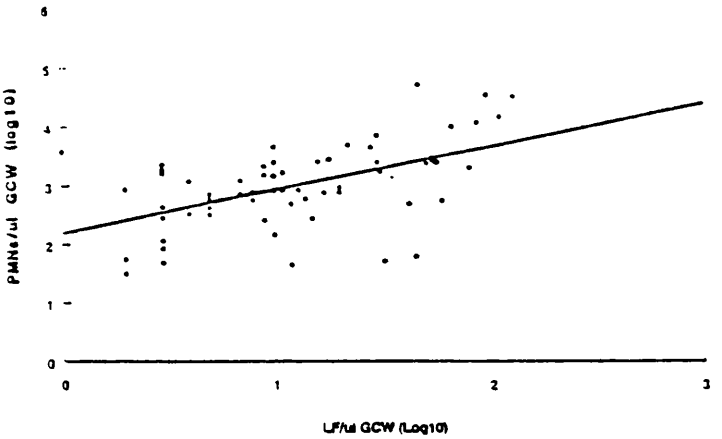

Fig. 1. PMNs/µl GCW as function of GCW LF levels (ng/µl GCW) in $n=63$ GCWs. Both variables are transformed to $\log_{10}$. The Pearson correlation coefficient ($r=0.531$, $p<0.001$) was determined and the best fitting line plotted by the least squares method (regression analysis).

GCW respectively). However, as GCW is a semiquantitative technique, GCW LF and PMN concentrations are directly proportional to their absolute amounts present within the crevice.

Our data demonstrate that GCF LF (ng/30 s sample) correlates positively with the degree of gingival inflammation ($r=0.418$, $p<0.001$, $n=71$) and pocket depth ($r=0.415$, $p<0.001$, $n=71$) (Table 1). LF is contained within the PMN secondary granules (Spitznagel et al. 1974) which also contain lysozyme and collagenase (Baggiolini et al. 1978). The latter are potent enzymes which are released in the crevice at the same time as LF during PMN degranulation and could contribute to the development of inflammation and cause surrounding tissue damage (Cimasoni 1983). The activity of such enzymes in the crevice is modulated by inhibitors like α1-antitrypsin (MWT:55 kD) and α2-macroglobulin (MWT:720 kD) present in the area (Adonogianaki et al. 1992). These inhibitors would be less effective if the PMN was closely adherent to its substrate (bacteria or epithelial cells) as the interface (PMN-substrate) would exclude proteins with a molecular weight greater than 50 kD (Wilton 1986). LF, which enhances PMN adhesiveness (Oseas et al. 1981), may thus have a synergistic effect on PMN enzymes and indirectly contribute to surrounding tissue damage. Moreover, LF could directly cause damage to host cells due to its strongly cationic charge. Therefore, although LF has properties beneficial to the host (antimicrobial), it also has potentially harmful features. LF is present in only trace amounts in serum (Hetherington et al. 1983). The positive correlation of GCF LF (ng/30 s) with GCF volume ($r=0.624$, $p<0.001$, $n=71$) (Table 1) suggests that a proportion of LF may be released by the PMN infiltrate present within the connective tissue underlying the junctional epithelium area and may be passively transferred into the crevice by GCF flow.

When the sites sampled for GCF were allocated into the three clinical groups (Table 2), gingivitis ($p=0.012$) and periodontitis ($p=0.002$) sites demonstrated elevated LF levels (ng/30 s) when compared to healthy sites. No significant difference however, could be observed between gingivitis and periodontitis sites although the latter did demonstrate a tendency for increasing LF levels. In this cross-sectional study, PD was used as the differentiating factor between gingivitis and periodontitis sites. PD, reflects the history, rather than the activity, of periodontal disease at a specific site and this may have a bearing on the inability of LF levels (ng/30 s) to differentiate periodontitis from gingivitis sites.

LF has been quantified previously in pooled GCF, and higher LF levels were obtained at gingivitis, periodontitis and localised periodontitis patients when compared to healthy controls (Friedman et al. 1983). However, as the methodologies applied in this and our study differ substantially comparison between the results is difficult.

Is LF a useful marker of crevicular PMNs? When the association between PMN numbers and LF levels in GCWs from discrete sites was examined a positive ($r=0.531$, $n=63$) and significant ($p<0.001$) association was obtained (Fig. 1). The moderately strong relationship between GCW LF and crevicular PMNs could be due to the LF contribution of degranulating PMNs within the gingival connective tissue. Alternatively, LF might reflect better the activated rather than the total PMNs present in the crevice.

When associations between the clinical indices and LF concentration or PMN numbers in GCWs were examined positive correlations were obtained (Table 3). Interestingly, LF in GCWs demonstrated stronger correlations with both GI and PD than did PMN numbers (Table 3). This suggests that PMN activation/degranulation may be a better test of periodontal disease status at a specific site rather than simply PMN numbers present in the crevice.

In conclusion, our data support the view that LF is a useful marker of PMN emigration and/or activation in the crevice. LF determination in GCF is more accurate and simpler to perform than the enumeration of PMNs using GCWs or styroflex strips. LF correlates well with the clinical indices of the sites sampled but further investigation of a longitudinal nature is required in order to assess its potential as a marker of periodontal disease activity.

Acknowledgements

The present study was supported by the Greater Glasgow Health Board, Research Support Group, RSG/END/9091/N.

Zusammenfassung

Laktoferrin im gingivalen Sulkus als Marker für polymorphkernige Leukozyten bei parodontalen Krankheiten

Um die Hypothese zu prüfen, daß Laktoferrin als Marker für krevikuläre, polymorphkernige Leukozyten (PMN) infrage kommen kann, wurden in dieser Studie die Laktoferrin (LF) Niveaus im krevikulären Sekret der Gingiva (GCF) untersucht und dargestellt. 2 Experimente wurden vorgenommen: a) zur Quantifizierung der gesamten LF (ng/30 s Probe) im GCF (krevikulären Gingivasekret) und; b) zur Korrelation der LF-Niveaus (ng/µl) und der PMN-Zahl (PMN/µl) bei krevi-

kulären Gingivaspülungen (GCW). Bei im ganzen 22 patienten wurden an 71 Stellen GCF entnommen. Von klinischen Indizes bei der Gingivitis (GI) und der Taschentiefe (PD) ausgehend, wurden diese Stellen in die drei klinischen Gruppen: 'gesund', 'Gingivitis' und 'Parodontitis' eingeteilt. Danach wurden bei 21 Patienten an weiteren 63 Stellen GCW's (krevikuläre Gingivaspülungen) vorgenommen. Das Vorkommen von LF in der GCW wurde durch eine Sandwich ELISA geprüft. In den GFW's wurden Gesamt- und differenzierte Leukozytenauszählungen vorgenommen. Die LF-Niveaus im GCF (ng/30 s) korrelierten positiv mit der GI ($r=0.418$, $p<0.001$), der PD ($r=0.415$, $p<0.001$) und dem GCF Volumen ($r=0.624$, $p<0.001$). Es zeigte sich, daß in Gingivitis- ($n=21$), und Parodontitisstellen ($n=24$) signifikant höhere ($p<0.05$) GCF LF Gesamtwerte vorkamen als in gesunden ($n=26$). Bei den LF-Werten in GCW's (ng/μl) waren stärkere Korrelationen zu klinischen Indizes vorhanden (GI: $r=0.452$, PD: $r=0.513$, $p<0.001$) als bei den PMN-Zahlenwerten (PMNs/μl) (GI: $r=0.279$, PD: $r=0.388$, $p<0.05$). LF-Werte korrelierten stark mit den PMNs in den GCWs ($r=0.531$, $p<0.001$) und bieten also einen einfachen und effektiven Marker krevikulärer PMN-Zahlen an.

Résumé

La lactoferrine du sillon gingival en tant que marqueur des leucocytes polynucléaires dans la maladie parodontale

Cette étude a examiné si les taux de lactoferrine (LF) du fluide créviculaire gingival (GCF) pouvaient servir de marqueur des leucocytes polynucléaires (PMN) du sillon gingival. La première expérience a été de calculer la quantité de LF présente dans le GCF en ng pour un échantillonage de 30 s. La seconde consistait à mettre en relation taux de LF (ng/μl) et nombre de PMN (PMNs/μl) dans des lavages créviculaires (GCW). Le GCF a été prélevé au niveau de 71 sites chez 22 patients. Ces sites ont été répartis en «sains», «gingivites» et «parodontites» suivant l'indice d'inflammation gingivale (GI) et la profondeur de poche au sondage (PD). Les GCWs ont également été prélevés au niveau de 63 sites chez 21 patients. Les teneurs en LF dans le GCF et les GCWs ont été mesurées par ELISA sandwich. Le nombre total de leucocytes et les comptages différentiels ont été effectués sur les GCWs. La LF présente dans le GCF était en relation positive avec le GI ($r=0.418$; $p<0.001$), la PD ($r=0.415$; $p<0.001$) et le volume de GCF ($r=0.624$; $p<0.001$). Les 21 sites avec gingivite et les 24 avec parodontite avaient davantage ($p<0.05$) de LF totale dans le GCF que les 26 sains. Dans les GCWs la LF montrait plus de corrélation avec les indices cliniques (GI: $r=0.452$; PD: $r=0.513$ et $p<0.001$) que ne le faisait le nombre de PMNs (GI: $r=0.279$; PD: $r=0.388$ et $p<0.05$). La LF était fort en corrélation avec les PMNs des GCWs ($r=0.531$ et $p<0.001$) et représente donc un marqueur simple et efficace du nombre de PMNs créviculaires.

References

Adonogianaki, E., Mooney, J. & Kinane, D. F. (1992) The ability of gingival crevicular fluid acute phase proteins to distinguish healthy, gingivitis and periodontitis sites. *Journal of Clinical Periodontology*, in press.

Arnold, R. R., Russell, J. E., Champion, W. J., Brewer, M. & Gauthier, J. J. (1982) Bactericidal activity of human lactoferrin: Differentiation from the stasis of iron deprivation. *Infection and Immunity* 35, 792–799.

Attström, R. & Egelberg, J. (1970) Emigration of blood neutrophils and monocytes into the gingival crevices. *Journal of Periodontal Research* 5, 48–55.

Baggiolini, M., Bretz, U. & Dewald, B. (1978) Subcellular localization of granulocyte enzymes. In: *Neutral proteases of human polymorphonuclear leukocytes*, eds. Havemann, K. & Janoff, A., pp. 3–17. Baltimore-Munich: Urban & Schwarzenberg.

Bartal, I., Padeh, S. & Passwell, J. H. (1987) Lactoferrin inhibits prostaglandin E_2 secretion by breast milk macrophages. *Pediatric Research* 21, 54–57.

Bennett, R. M. & Kokocinski, T. (1978) Lactoferrin content of peripheral blood cells. *British Journal of Haematology* 39, 509–521.

Bentwood, B. J. & Henson, P. M. (1980) The sequential release of granule constituents from human neutrophils. *The Journal of Immunology* 124, 855–862.

Britigan, B. E., Hassett, D. J., Rosen, G. M., Hamill, D. R. & Cohen, M. S. (1989) Neutrophil degranulation inhibits potential hydroxyl-radical formation. Relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. *Biochemical Journal* 264, 447–455.

Cimasoni, G. (1983) *Crevicular fluid updated*, 2nd edition, pp. 82–102. Basel: Karger.

Cimasoni, G. & Giannopoulou, C. (1988) Can crevicular fluid component analysis assist in diagnosis and monitoring periodontal breakdown? In: *Periodontology today*. Int. Congr., Zurich 1988, pp. 260–270. Basel: Karger.

Curtis, M. A., Gillett, I. R., Griffiths, G. S., Maiden, M. F. J., Sterne, J. A. C., Wilson, D. T., Wilton, J. M. A. & Johnson, N. W. (1989) Detection of high-risk groups and individuals for periodontal diseases: Laboratory markers from analysis of gingival crevicular fluid. *Journal of Clinical Periodontology* 16, 1–11.

Ebersole, J. L., Taubman, M. A., Smith, D. J. & Goodson, J. M. (1984) Gingival crevicular fluid antibody to oral microorganisms I. Method of collection and analysis of antibody. *Journal of Periodontal Research* 19, 126–132.

Falkon, J. & Gallin, J. I. (1986) Neutrophil granules in health and disease. *Journal of Allergy Clinical Immunology* 77, 653–662.

Fine, D. H. & Mandel, I. D. (1986) Indicators of periodontal disease activity: an evaluation. *Journal of Clinical Periodontology* 13, 533–546.

Friedman, S. A., Mandel, I. D. & Herrera, M. S. (1983) Lysozyme and lactoferrin quantitation in the crevicular fluid. *Journal of Periodontology* 54, 347–350.

Gillett, I. R., Johnson, N. W., Curtis, M. A., Griffiths, G. S., Sterne, J. A. C., Carman, R. J., Bampton, J. L. M. & Wilton, J. M. A. (1990) The role of histopathology in the diagnosis and prognosis of periodontal diseases. *Journal of Clinical Periodontology* 17, 673–684.

Griffiths, G. S., Curtis, M. A. & Wilton, J. M. A. (1988) Selection of a filter paper with optimum properties for the collection of gingival crevicular fluid. *Journal of Periodontal Research* 23, 33–38.

Henson, P. M. (1971a) The immunologic release of constituents from neutrophil leukocytes. I. The role of antibody and complement on nonphagocytosable surfaces or phagocytosable particles. *The Journal of Immunology* 107, 1535–1546.

Henson, P. M. (1971b) The immunologic release of constituents from neutrophil leukocytes II. Mechanisms of release during phagocytosis, and adherence to nonphagocytosable surfaces. *The Journal of Immunology* 107, 1547–1557.

Hetherington, S. V., Spitznagel, J. K. & Quie, P. G. (1983) An enzyme-linked immunoassay (ELISA) for measurement of lactoferrin. *Journal of Immunological Methods* 65, 183–190.

Kinane, D. F. & Davies, R. M. (1990) Periodontal manifestations of systemic disease. In: *Oral manifestations of systemic disease*, eds. Jones, H. & Mason, D. K., pp. 512–536. London: Saunders.

Kowashi, Y., Jaccard, F. & Cimasoni, G. (1980) Sulcular polymorphonuclear leucocytes and gingival exudate during experimental gingivitis in man. *Journal of Periodontal Research* 15, 151–158.

Lamster, I. B., Oshrain, R. L. & Gordon, J. M. (1986) Enzyme activity in human gingival crevicular fluid: considerations in data reporting based on analysis of individual crevicular sites. *Journal of Clinical Periodontology* 13, 799–804.

Lobene, R., Weatherford, T., Ross, W., Lamm, R. & Manaker, L. (1986) A modified gingival index for use in clinical trials. *Clinical Preventive dentistry* 8, 3–6.

Osese, R., Yang, H.-H., Bashner, R. L. & Boxer, L. A. (1981) Lactoferrin: A promoter of polymorphonuclear leukocyte adhesiveness. *Blood* 57, 939–945.

Pelus, L. M., Broxmeyer, H. E., Kurland, J. I. & Moore, M. A. S. (1979) Regulation of macrophage and granulocyte proliferation. *Journal of Experimental Medicine* 150, 277–292.

Skapski, H. & Lehner, T. (1976) A crevicular washing method for investigating immune

components of crevicular fluid in man. *Journal of Periodontal Research* 11, 19–24.
Smith, Q. T. & Geegan, S. J. (1991) Repeated measurement of crevicular fluid parameters at different sites. *Journal of Clinical Periodontology* 18, 171–176.
Spitznagel, J. K., Dalldorf, F. G., Leffell, M. S., Folds, J. D., Welsh, I., R. H., Cooney, M. H. & Martin, L. E. (1974) Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. *Laboratory Investigation* 30, 774–785.
Taichman, N. S., Tsai, C.-C., Baehni, P. C., Stoller, N. & McArthur, W. P. (1977) Interaction of inflammatory cells and oral microorganisms IV. In vitro release of lysosomal constituents from polymorphonuclear leukocytes exposed to supragingival and subgingival plaque. *Infection and Immunity* 16, 1013–1023.
Thurre, C., Robert, M., Cimasoni, G. & Baehni, P. (1984) Gingival sulcular leukocytes in periodontitis and in experimental gingivitis in humans. *Journal of Periodontal Research* 19, 457–468.
Wilton, J. M. A. (1986) Crevicular neutrophils: protective or damaging? In: *Borderland between caries and periodontal disease III*, eds. Lehner, T. & Cimasoni, G. pp. 71–85. Geneve: Editions Medicine et Hygiene.
Wilton, J. M. A., Griffiths, G. S., Curtis, M. A., Maiden, M. F. J., Gillett, I. R., Wilson, D. T., Sterne, J. A. C. & Johnson, N. W. (1988) Detection of high-risk groups and individuals for periodontal diseases. Systemic predisposition and markers of general health. *Journal of Clinical Periodontology* 15, 339–346.
Wright, D. G. & Gallin, J. I. (1979) Secretory responses of human neutrophils: Exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *The Journal of Immunology* 123, 285–294.
Zucali, J. R., Broxmeyer, H. E., Levy, D. & Morse, C. (1989) Lactoferrin decreases monocyte-induced fibroblast production of myeloid colony-stimulating activity by suppressing monocyte release of interleukin-1. *Blood* 74, 1531–1536.

Address:

D. F. Kinane
Periodontal Unit
Department of Oral Medicine & Pathology
Glasgow Dental Hospital and School
378 Sauchiehall Street
Glasgow, G2 3JZ
Scotland, UK

Immunocytochemical characterization of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during human experimental gingivitis

Denis F. Kinane, Eva Adonogianaki, Naureen Moughal, F. Peter Winstanley, John Mooney and Martin Thornhill*

Dept. Oral Medicine and Pathology, University of Glasgow Dental School, *Dept. of Rheumatology, UMDS, London, U.K.

Kinane DF, Adonogianaki E, Moughal N, Winstanley FP, Mooney J, Thornhill M: Immunocytochemical characterization of cellular infiltrate, related endothelial changes and determination of GCF acute-phase proteins during human experimental gingivitis. J Periodont Res 1991; 26: 236–238.

Key words: experimental gingivitis – immunocytochemistry – acute-phase proteins – gingival crevicular fluid

Accepted for publication 15 February 1991

Introduction

During the initiation of inflammation, vascular endothelium is activated by a variety of molecules which increase the permeability of vessels and regulate the passage of leukocytes from the bloodstream into the inflamed tissues. Of the molecules capable of acting on the endothelial cells, interleukin-1 (IL-1), an inflammatory cytokine, has been detected in inflamed gingiva (1). IL-1 upregulates expression of endothelial leucocyte adhesion molecules-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1), which are ligands for adhesion molecules on leukocytes (integrins), and thus influence gingival leukocyte infiltration.

α2-macroglobulin (α2-M) and α1-antitrypsin (α1-AT) together comprise 90% of the serum protease inhibitors (2) and have been identified in gingival crevicular fluid (GCF) from diseased sites at about 75% of their serum levels (3). Transferrin (TF) is a serum-derived iron-binding glycorprotein (4) which in GCF might function as an antibacterial agent by producing an iron-limiting environment (5). TF has been reported in GCF at levels similar to those of α2-M and α1-AT (3). Recent evidence suggests that, in addition to their classical roles, these proteins can modulate the immune responses via several pathways: α2-M binds important effector molecules such as cytokines and cell receptors which inhibit immunological reactions (6); α1-AT has been implicated in the inhibition of lymphocyte responses, complement activation and neutrophil migration (7); and TF is involved in T-cell transformation and macrophage activation (8).

The dynamics of these acute-phase proteins, and the cytokine IL-1 in GCF, were compared with leukocyte infiltration and expression of the endothelial cell adhesion molecules ICAM-1 and ELAM-1, during 21-day experimental gingivitis episode (9).

Material and Methods

Twelve healthy students stopped toothbrushing for 21 d. At baseline (d 0), and after 4, 7, 11, 14, 17 and 21 d of undisturbed plaque accumulation and following the reinstitution of oral hygiene procedures (d 28 and 35), clinical changes were recorded using a Plaque Index (10) and a modified Gingival Index (11), and GCF was sampled using Whatman grade-4 paper strips (2 × 13 mm) (12) for 30 seconds. Quantification of GCF volume was performed using the Periotron 6000 (Harco). The strips were eluted into 1 ml of phosphate-buffered saline and 200 μl aliquots were analyzed for the α2-M, α1-AT and TF. For the IL-1 bioassay, GCF was eluted from the paper strips by incubation for 30 minutes at 4°C in 150 μl of RPMI 1640 medium with supplements. Indirect competitive immunoassays were developed for the quantification of α2-M and TF. α1-AT was assayed using a double-antibody sandwich assay. For each individual, a mean values was obtained for each sampling incidence for α2-M, α1-AT and TF levels (in ng per 30-s sample).

IL-1 activity was determined by bioassay using

the IL-1-sensitive cell line D10(N4)M (13) except that cell proliferation was determined by a colorimetric method (14). The potency of the stimulatory activity in the eluate was determined from dose-response curves obtained with a recombinant human DNA-derived IL-1 beta (rhIL-1β) standard, using the computer program ALLFIT (15). Gingival biopsies (2 × 2 mm) were taken from the first molar buccal gingiva on d 0, 7, 14 and 21 and embedded in Tissue Tek, snap frozen in liquid nitrogen after which serial 8 μm thick sections were cut. Frozen sections were immunoperoxidase-stained using a panel of monoclonal antibodies for Langerhans cells, T-helper, T-suppressor, primed T (CD45RO), pan-T and B cells, monocyte/macrophages, neutrophils, HLA-DR, ICAM-1 and ELAM-1. Leukocytes in the sections were counted at ×400 magnification in defined areas. Langerhans cells and other infiltrating cells within the epithelium were counted and the area of sectioned oral epithelium (AOE) determined, using computer assisted image analysis. Three adjacent areas within each section were counted and the mean number of positive cells per biopsy was calculated and expressed as either cells per 0.1 mm^2 of connective tissue or of AOE. HLA-DR, ICAM-1 and ELAM-1 were scored using a grading system for specified areas of the section.

Results

Following withdrawal of oral hygiene procedures the plaque index rose rapidly and was followed by the gingival inflammatory index. GCF IL-1 peaked within 7 d of the inflammatory episode ($p < 0.05$). α2-M, α1-AT, TF and GCF volume increases matched the gingival inflammation index. α2-M levels throughout the study were markedly higher than the levels of α1-AT ($p < 0.05$).

The term sulcular epithelium (SE) was used to include both the JE and oral sulcular epithelium. Langerhans cell (LC) numbers increased from baseline and peaked at d 14 ($p = 0.03$; Mann-Whitney). HLA-DR staining increased from baseline and peaked at d 7, after which the HLA-DR$^-$ cellular infiltrate started to fall, reaching baseline levels by d 21. Pan-T cells and PMN also peaked at d 7 in the SE, then reduced as inflammation progressed. Expression of vascular ICAM-1 and ELAM-1 increased at or before d 7, which coincided with the peak in IL-1 levels and T-cell infiltration. ICAM-1 expression in junctional epithelial keratinocytes peaked at d 7 and exhibited a gradient effect in the density of ICAM-1 from the crevicular towards the basal region of the epithelium.

Discussion

During the initiation of gingival inflammation many immune and inflammatory events take place at different time points. IL-1 appears to peak at d 7, as does upregulation of ELAM-1, ICAM-1, HLA-DR and leukocyte infiltration. The changes in acute-phase proteins appear to follow changes in the inflammatory index and probably reflect the changes in vascular permeability. α2-M levels throughout the study were markedly higher than the levels of α1-AT and may reflect the fact that α2-M can be locally produced by resident cells or infiltrating macrophages (16).

Sulcular neutrophil numbers decreased as gingivitis progressed and only recovered to baseline levels at d 21, suggesting PMN movement into the gingival crevice, presumably by chemotaxis. The spatial and temporal changes in PMN numbers illustrate the difficulty in interpretation using sequential biopsies. The flux of cells into the crevice, or back into the connective tissue, or arriving from the blood vessels, cannot easily be determined without time-lapse video analysis. However, immunocytochemistry and GCF ELISA techniques are valuable tools in the investigation of immune and inflammatory processes in experimental gingivitis and may elucidate the role of specific cells and soluble factors in the initiation and pathogenesis of periodontal disease.

References

1. Honig J, Rordorf-Adm C, Siegmund C, Wiedemann W, Erard F. Interleukin-1 beta (IL-1β) concentration in gingival tissue from periodontitis patients and healthy control subjects. *J Periodont Res* 1989; 24: 362–367.
2. Sandholm L. Proteases and their inhibitors in chronic inflammatory periodontal disease. *J Clin Periodontol* 1986; 13: 19–32.
3. Scheinken HA, Genco RJ. Gingival fluid and serum in periodontal diseases I. Quantitative study of immunoglobulins, complement components and other plasma proteins. *J Periodontol* 1977; 48: 772–777.
4. Aissen P. Transferrins. In: Jacobs A, Worwood M, eds. *Iron in biochemistry and medicine*. London and New York: Academic Press, 1980: 29–71.
5. Curtis MA, Gillett IR, Griffiths GS, et al. Detection of high-risk groups and individuals for periodontal diseases: Laboratory markers from analysis of gingival crevicular fluid. *J Clin Periodontol* 1989; 16: 1–11.
6. James K. Interactions between cytokines and α2-macroglobulin. *Immunol Today* 1990; 11: 163–166.
7. Breit SN, Penny R. The role of α1 protease inhibitor (α1 antitrypsin) in the regulation of immunologic and inflammatory reactions. *Aust NZ J Med* 1980; 10: 449–453.
8. Brock JH, Mainou-Fowler T. The role of iron and transferrin in lymphocyte transformation. *Immunol Today* 1983; 4: 347–351.
9. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol* 1965; 36: 177–187.
10. Silness P, Loe H. Periodontal disease in pregnancy II. Cor-

relation between oral hygiene and periodontal condition. *Acta Odontologica Scandinavica* 1964; 22: 121–135.
11. Lobene RR, Weatherford T, Ross NM. A modified gingival index for use in clinical trials. *Clin Prev Dent* 1986; 8: 3–9.
12. Griffiths GS, Wilton JMA, Curtis MA, et al. Detection of high-risk groups and individuals for periodontal diseases. Clinical assessment of the periodontium. *J Clin Periodontol* 1988; 15: 403–410.
13. Hopkins SJ, Humphries M. Simple, sensitive and specific bioassay for Interleukin 1. *J Immunol Methods* 1989; 120: 271–276.
14. Mossman T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55–60.
15. De Lean A, Munson PJ, Rodbard D. Simultaneous analysis of families of sigmoid curves: Application to bioassay, radioligand assay, and physiological dose response curves. *Am J Physiol* 1978; 235: E97.
16. Conducci I, Cimasoni G, Rey M, Baehni P. In vitro synthesis of α2-macroglobulin by human gingival fibroblasts. *Arch Oral Biol* 1988; 33: 407–412.

Address:

Dr. D.F. Kinane
Dept. of Oral Medicine and Pathology
University of Glasgow Dental Hospital and School
378 Sauchiehall Street
Glasgow G2 3JZ
U.K.

Archs oral Biol. Vol. 37, No. 2, pp. 153–156, 1992
Printed in Great Britain.

0003-9969/92 $5.00 + 0.00

SHORT COMMUNICATION

BIOASSAY OF INTERLEUKIN 1 (IL-1) IN HUMAN GINGIVAL CREVICULAR FLUID DURING EXPERIMENTAL GINGIVITIS

D. F. KINANE,[1] F. P. WINSTANLEY,[2] E. ADONOGIANAKI[1] and N. A. MOUGHAL[1]

[1]Department of Oral Medicine and Pathology, University of Glasgow Dental School, and [2]Department of Pathological Biochemistry, Glasgow Royal Infirmary, Glasgow, U.K.

(Accepted 22 August 1991)

Summary—The cytokine IL-1 was demonstrated in crevicular fluid during a 14- and 21-day experimental gingivitis in healthy human volunteers. A sensitive and specific bioassay allowed detection of biologically active IL-1 at levels ranging from 0.18 ng/μl at baseline to 1.70 ng/μl in inflamed gingiva. Levels of IL-1 increased rapidly with plaque accumulation and in advance of the subsequent gingival inflammation, peaking within 7 days of the start of gingivitis. As changes in IL-1 were detected before clinically recognizable gingival changes, IL-1 may have potential as an early marker of gingival inflammatory changes.

Key words: interleukin 1, experimental gingivitis.

IL-1 is a cytokine found in two forms, IL-1α and IL-1β, which have been shown to be intimately involved in the initiation and regulation of the inflammatory response (Dinarello, 1984). Although structurally these molecules differ, the potency and activity of both are virtually identical and they bind to the same receptor with equal affinity (March *et al.*, 1985; Sims *et al.*, 1988).

Oppenheim, Charon and Luger (1982) and Charon *et al.* (1982) have reported significant increases in IL-1 from inflamed sites in periodontitis. These findings, however, were based on the detection of IL-1 in a thymocyte proliferation assay that is not specific as it may also respond to other cytokines such as IL-2, -6 and tumour necrosis factor-α (Lotz *et al.*, 1988; Ranges *et al.*, 1988). In addition, thymocyte proliferation assays are relatively insensitive to IL-1. More recently, immunoassays have been used to demonstrate IL-1β in gingival tissues from patients with chronic periodontitis (Hönig *et al.*, 1989) and both IL-1α and IL-1β in GCF from periodontally diseased sites (Masada *et al.*, 1990). Although immunoassays can be very sensitive and specific for detecting molecules that bear particular epitopes, the molecule's biological function is uncertain, i.e. it could be bound to an inhibitor or in an other inactive form. We have now examined the changes in IL-1 concentration in GCF, using a specific and sensitive bioassay for both IL-1α and IL-1β, and the experimental gingivitis model of Löe, Theilade and Jensen (1965) which is ideally suited to investigations of the composition of GCF during inflammatory episodes.

Abbreviations: GCF, gingival crevicular fluid; IL, interleukin.

Two groups of six dental students aged between 20 and 21 yr (8 male, 4 female), with no evidence of periodontal disease and unremarkable medical histories, volunteered for the study. All had more than 26 teeth, a high standard of oral hygiene and healthy gingiva. For 2 months before the experiments the subjects were regularly examined and their oral hygiene monitored to achieve maximal gingival health. After baseline values had been measured the volunteers were instructed to stop all oral hygiene procedures. One group of six stopped for 14 days and the other for 21 days, after which they were given a thorough professional prophylaxis, told to start their normal oral hygiene procedures again and checked 1 and 2 weeks later. Clinical measurements and GCF samples were taken at baseline and then at 4-day intervals for the 14-day group and at 7-day intervals for the 21-day group, from the same two sites in each patient—the lower right lateral incisor, distobuccal aspect and the lower right central incisor, distobuccal aspect. Plaque accumulation was assessed by the Plaque Index of Silness and Löe (1964) and the Lobene *et al.* (1986) modified Gingival Index was used for non-invasive evaluation of the early visible changes in severity and extent of gingivitis. One examiner recorded the clinical indices throughout the experiment. GCF was collected with 2 × 13-mm strips of Whatman grade 4 paper after the crevice had been gently air-dried and supragingival plaque removed. The paper strip was inserted into the crevice until mild resistance was felt, and left for 30 s. Care was taken to avoid mechanical injury. After collection the paper strip was transferred at the chairside to a Periotron 6000 (Harco, Winnipeg, Canada), for quantification of the volume collected. The strips were then stored at −30°C until further processing.

Table 1. Plaque Index (PI), modified Gingival Index (MGI), GCF and IL-1 for the six subjects during the 14-day experimental gingivitis study

	Days													
Indices	0	(SE)	4	(SE)	7	(SE)	11	(SE)	14	(SE)	21	(SE)	28	(SE)
PI	0.17	0.11	1.58	0.25	1.58	0.14	1.9	0.19	2.7	0.14	0.5	0.22	0.5	0.22
MGI	0.17	0.11	0.17	0.11	1.33	0.25	2.17	0.23	2.8	0.13	2.5	0.22	0.33	0.14
GCF(μl)	0.21	0.02	0.23	0.03	0.36	0.06	0.41	0.03	0.54	0.04	0.43	0.05	0.36	0.06
IL-1 (ng/μl)	0.19	0.05	1.30	0.36	1.57	0.48	0.78	0.16	0.95	0.23	0.19	0.05	0.20	0.07

Means and SE are shown.

GCF was eluted from the paper strips by incubation for 30 min at 4°C in 150 μl of RPMI 1640 medium supplemented with 5% fetal calf serum, 30 μg/ml gentamicin and 2.5 μg/ml amphotericin B. After elution, the paper strip was removed and the eluate was centrifuged for 2 min in a microfuge to remove any particulate matter. The supernatant was stored frozen at −20°C for later assay. IL-1 activity was bioassayed using the IL-1 sensitive cell line D10(N4)M (Helle, Boeile and Aarden, 1988; Hopkins and Humphries, 1989) with the modification that cell proliferation was determined by a colorimetric method with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Mossman, 1983). The potency of the stimulatory activity in the eluate was determined from dose–response curves obtained with a recombinant human DNA-derived IL-1β (*rhil*-1β) standard using the computer program ALLFIT (De Lean, Munson and Rodbard, 1978). Adding enough anti-IL-1β serum [a sheep polyclonal serum (S77β); gift of Dr A. Shaw, Glaxo, Geneva] to 20 μl of selected eluates, to neutralize completely the activity of 100 pg of *rhil*-1β, inhibited at least 70% of the stimulatory activity. This bioassay is particularly sensitive and specific for IL-1 and thus the remaining activity was presumed to be associated with IL-1α.

The mean Gingival Indices, Plaque Indices, GCF volume and IL-1 levels for the six subjects during the 14-day experimental gingivitis are given in Table 1. After abstaining from oral hygiene, dental plaque accumulated rapidly. This was followed by an increase in local gingival inflammation that lagged behind the detectable increase in plaque. The IL-1 concentration increased rapidly with plaque accumulation and in advance of the subsequent gingival inflammation. At day 14, when regular oral hygiene procedures were resumed, the Plaque Index rapidly returned to baseline. The modified Gingival Index (inflammation index) followed the rise and fall in the plaque accumulation index, but lagged behind it by a few days. The average IL-1 level for this group of subjects had started to drop well before normal oral hygiene was started again, and returned to baseline levels ahead of the inflammation index. A similar series of changes was found with the 21-day gingivitis (Table 2). Regression analysis was done with the IL-1 levels, Plaque Index and modified Gingival Index for each subject summed over the seven sampling intervals for the 14-day gingivitis and the four sampling intervals for the 21-day experiment (Table 3) (Matthews *et al.*, 1990). Plaque and IL-1 levels were both positively correlated with the modified Gingival Index, although the relationship between Plaque Index and IL-1 was less strong.

Although IL-1 was originally considered to be a product of mononuclear phagocytes, recent evidence suggests that keratinoctes and gingival fibroblasts can also produce IL-1 in response to stimulation by bacterial components (Walsh, Seymour and Powell, 1985; Hanazawa *et al.*, 1985, 1988; Takada *et al.*, 1991). The accumulation of dental plaque might provide the appropriate trigger for gingival cells or monocyte/macrophages to produce IL-1, which would then propagate an inflammatory response. In this experimental gingivitis the increase in IL-1 concentration in the crevicular fluid was found before there was observable inflammation of the gingiva, but closely followed the Plaque (accumulation) Index. Whereas the plaque and inflammation indices continued to increase whilst oral hygiene remained withdrawn, the concentration of IL-1 began to decline after day 7. This early decline may be a normal regulation of IL-1 production, or due to inhibition or degradation by host or bacterial factors. Non-specific inhibition of IL-1 bioactivity might result from proteolytic digestion by phagocyte and microbial enzymes present in the crevice fluid (Sandholm, 1986). In the gingival sulcus IL-1 might also be affected by an inhibitor produced by human gingival epithelial cells (Walsh *et al.*, 1987). The early rise of IL-1 concentration suggests its involvement in the initiation and propagation of the gingivitis.

Although there was minimal intra-individual variation in our clinical and biochemical data (not shown), we found a wide intersubject variation in the quantity of crevicular IL-1. Whereas most of the

Table 2. Plaque Index (PI), modified Gingival Index (MGI), GCF and IL-1 for the six subjects during the 21-day experimental gingivitis study

	Days							
Indices	0	(SE)	7	(SE)	14	(SE)	21	(SE)
PI	0.25	0.5	1.6	0.5	2.0	0.1	2.2	0.4
MGI	0.0	0.0	1.0	0.7	2.0	0.1	2.2	0.4
GCF(μl)	0.23	0.08	0.2	0.05	0.15	0.02	0.26	0.06
IL-1(ng/μl)	0.47	0.19	1.74	0.69	1.05	0.14	0.54	0.11

Means and SE are shown.

Table 3. Regression analysis of experimental indices

Comparison	r	p
PI versus IL-1	0.59	0.045
PI versus MGI	0.72	<0.01
IL-1 versus MGI	0.76	0.017

PI, Plaque Index; MGI, modified Gingival Index.

subjects developed a substantial rise in crevicular IL-1 activity, which peaked within 7 days of withdrawing oral hygiene, two subjects within the 14 day study showed only slight crevicular IL-1 activity throughout the study. The heterogeneous IL-1 responses of these subjects might reflect individual variation in plaque accumulation or variation in subsequent inflammation due to plaque. Alternatively, it might be a result of an inherent variation in the ability to produce IL-1 at this site. The lack of correlation between the Plaque Index and IL-1 in these subjects suggests that plaque accumulation *per se* is not the major determining factor in gingival IL-1 production. The positive correlation between the Plaque Index and the modified Gingival Index (Table 3) is consistent with previous studies based on the experimental gingivitis model (Löe *et al.*, 1965). The overall correlation between IL-1 and the inflammatory index supports the proposed implication of IL-1 in gingival inflammation.

IL-1α and -β have recently been detected in larger amounts than normal from sites manifesting active periodontitis (Masada *et al.*, 1990). In the light of this, our findings of a peak and then a drop to baseline levels in the first few weeks of a developing inflammatory episode require further discussion. The experimental gingivitis model allows examination of early changes during the development of periodontal disease. A gradual increase in basal levels of IL-1 may take place during the long time course of developing chronic gingivitis and periodontitis. The early peak in IL-1 levels reported here is consistent with the 'burst hypothesis' of periodontal disease progression, and if this dramatic change in IL-1 levels occurs in periodontitis sites it could be an indicator of incipient breakdown at those sites. IL-1 is one of the factors known to stimulate bone resorption and collagenase production by fibroblasts, both of which are features of periodontal destruction (Gowen *et al.*, 1983; Heath *et al.*, 1985; Postlethwaite *et al.*, 1983). In addition, as changes in IL-1 levels were detected well in advance of clinically recognizable gingival changes, IL-1 may have a role as an early marker of gingival inflammatory changes.

Acknowledgements—The *rhil*-1β and anti-IL-1β serum were kind gifts of Dr A. Shaw, Glaxo Institute for Molecular Biology, Geneva, Switzerland. The D10(N4)M cell line was provided by Dr S. Hopkins, University of Manchester, U. K. Dr F. P. Winstanley was supported by funds from the Scottish Hospitals Endowment Research Trust. The encouragement of Dr A. Shenkin, Glasgow Royal Infirmary, was appreciated.

REFERENCES

Charon J., Luger T., Mergenhagen S. and Oppenheim J. (1982) Increased thymocyte activating factor in human gingival fluid during gingival inflammation. *Infect. Immun.* **38**, 1190–1195.

De Lean A., Munson P. J. and Rodbard D. (1978) Simultaneous analysis of families of sigmoid curves: application to bioassay, radioligand assay, and physiological dose response curves. *Am. J. Physiol.* **235**, E97.

Dinarello C. A. (1984) Interleukin-1. *Rev. Infect. Dis.* **6**, 51–95.

Gowen M., Wood D. D., Ihrie E. J., McGuire M. K. B. and Russell R. G. G. (1983) An interleukin-1 like factor stimulates bone resorption *in vitro*. *Nature* **306**, 378–380.

Hanazawa S., Makada K., Ohmori Y., Miyoshi T., Amano S. and Kitano S. (1985) Functional role of Interleukin 1 in periodontal disease: induction of Interleukin 1 production by *Bacteroides Gingivalis* lipopolysaccharide in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. *Infect. Immun.* **50**, 262–270.

Hanazawa S., Hirose K., Ohmori Y., Amano S. and Kitano S. (1988) *Bacteroides Gingivalis* fimbriae stimulate production of Thymocyte-Activating Factor by human gingival fibroblasts. *Infect. Immun.* **56**, 272–274.

Heath J. V., Saklatvala J., Meikle M. C., Atkinson S. J. A. and Reynolds J. J. (1985) Pig interleukin-1 (catabolin) is a potent stimulator of bone resorption. *Calc. Tiss. Int.* **37**, 95–97.

Helle M., Boeile L. and Aarden L. A. (1988) Functional discrimination between Interleukin 6 and Interleukin 1. *Eur. J. Immun.* **18**, 1535–1540.

Hönig J., Rordorf-Adam C., Siegmund C., Wiedemann W. and Erard F. (1989) Interleukin-1 beta (IL-1β)concentration in gingival tissue from periodontitis patients and healthy control subjects. *J. periodont. Res.* **24**, 362–367.

Hopkins S. J. and Humphries M. (1989) Simple, sensitive and specific bioassay for Interleukin 1. *J. Immunol. Meth.* **120**, 271–276.

Lobene R., Weatherford T., Ross W., Lamm R. and Menaker L. (1986) A modified gingival index for use in clinical trials. *Clin. Prev. Dent.* **8**, 3–6.

Löe H., Theilade E. and Jensen S. B. (1965) Experimental gingivitis in man. *J. Periodont.* **36**, 177–187.

Lotz M., Jirik F., Kabouridis P., Tsoukas P. C., Hirano T., Kishimoto T. and Carson D. A. (1988) B cell stimulatory factor 2/interleukin 6 is a co-stimulant for human thymocytes and T lymphocytes. *J. exp. Med.* **167**, 1253–1259.

March C. J., Mosley B., Larsen A., Cerretti D. P., Braedt G., Price V., Gillis S., Henney C. S., Kronheim S. R. and Grabstein K. (1985) Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* **315**, 641–647.

Masada M. P., Persson R., Lee S. W., Page R. C. and Allison A. C. (1990) Measurement of gingival interleukin-1α and -1β in gingival crevicular fluid: implications for the pathogenesis of periodontal disease. *J. periodont. Res.* **25**, 156–163.

Matthews J. N. S., Altman D. G., Campbell M. J. and Royston P. (1990) Analysis of serial measurements in medical research. *Br. med. J.* **300**, 230–235.

Mossman T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* **65**, 55–60.

Oppenheim J. J., Charon J. A. and Luger T. A. (1982) Evidence for *in vivo* inflammatory role of interleukin 1 (IL-1). *Transplant. Proc.* **14**, 553–555.

Postlethwaite A. E., Lachman L. B., Mainardi C. L. and Kang A. H. (1983) Interleukin-1 stimulation of collagenase production by cultured fibroblasts. *J. exp. Med.* **157**, 801–806.

Ranges G. E., Zlotnik A., Espevik T., Dinarello C. A., Cerami A. and Palladino Jr M. A. (1988) Tumour necrosis factor α/cachectin is a growth factor for thymocytes. Synergistic interactions with other cytokines. *J. exp. Med.* **167**, 1472–1476.

Sandholm L. (1986) Proteases and their inhibitors in chronic inflammatory periodontal disease. *J. clin. Periodont.* **13**, 19–32.

Silness J. and Löe H. (1964) Periodontal disease in pregnancy. *Acta Odont. scand.* **22**, 121–135.

Sims J. E., March C. J., Cosman D., Widmer M. B., MacDonald H. R., McMahan C. J., Grubin C. E., Wignall J. M., Jackson J. L. and Call S. M. (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* **241**, 585–589.

Takada H., Mihara J., Morisaki I. and Hamada S. (1991) Induction of Interleukin-1 and -6 in human gingival fibroblast cultures stimulated with Bacteroides lipopolysaccharides. *Infect. Immun.* **59**, 295–301.

Walsh L. J., Seymour G. J. and Powell R. N. (1985) The *in vitro* effect of retinol on human gingival epithelium— II. Modulation of Langerhans cell markers and interleukin-1 production. *J. invest. Derm.* **85**, 501–506.

Walsh L. J., Lander P. E., Seymour G. J. and Powell R. N. (1987) Isolation and purification of ILS, and interleukin 1 inhibitor produced by human gingival epithelial cells. *Clin. exp. Immunol.* **68**, 366–374.

Heterogeneity and Selective Localisation of T Cell Clones in Human Skin and Gingival Mucosa

Denis F. Kinane, Sabeeha N. Karim[2], Jennifer J. Garioch[2], Adnan T. Al Badri[1], Naureen Moughal and Robert B. Goudie[1]

Dept. of Periodontology, University of Glasgow Dental School, [1]Dept. of Pathology, Royal Infirmary, Glasgow, Scotland and [2]Dept. of Dermatology, St. Mary's Hospital, London, U.K.

Running title: T cell clones in human skin and gingiva

Address for correspondence: Dr. D.F. Kinane,
Periodontology Department,
Glasgow Dental Hospital and School,
378 Sauchiehall Street,
Glasgow G2 3JZ, UK.

Key words: T cells, TCRγ genes, polymerase chain reaction, adhesion molecules.

Kinane DF, Karim SN, Garioch JJ, Al Badri AT, Moughal N, Goudie RB. Heterogeneity and Selective Localisation of T Cell Clones in Human Skin and Gingival Mucosa. J Periodont Res 1992; 00: 000-000.

Endothelial leukocyte adhesion molecule 1 (ELAM-1) and Intercellular adhesion molecule 1 (ICAM-1) are adhesion molecules which regulate cellular traffic into inflamed areas and both are present in clinically 'healthy' gingiva (1,2). It has recently been reported that the cutaneous lymphocyte-associated antigen, for which the monoclonal antibody HECA 452 is specific, is constitutively expressed in a subset of skin-homing memory T cells which predominate in normal and chronically inflamed skin (3). These skin-homing T cells bind to ELAM-1 which is expressed in skin (4) and gingiva (1,2). The marked lymphocytic infiltrate noted even in clinically healthy gingiva is intriguing, particularly the characteristic location of suppressor T cells and the possibility they have a role in gingival immune surveillance (2). To investigate whether there is selective cutaneous localisation of corresponding skin-homing T cell clones we have compared T cell receptor (TCR) gene rearrangement profiles in

samples of normal human skin, in normal gingival mucosa and in peripheral blood from the same subjects. T cell clones can be distinguished from each other by the antigen-binding domains of the TCRs (5) and by TCR gene rearrangements which include clone-specific hypervariable junctional regions (6). TCRγ genes are understood to be rearranged in all T cells including those which express αβ TCRs (7) and can act as clonal markers in small tissue samples (8). We have applied the polymerase chain reaction (PCR) to amplify the V-J junctions of TCRγ gene rearrangements and have separated the amplification products by high resolution electrophoresis to detect clone-related size differences. These differences result from variation in rearrangement breakpoints in the Vγ and Jγ genes and from insertion of uncoded N region nucleotides where the Vγ and Jγ genes join (6,8,9). Each of the four primer combinations used in the present study may give rise to 20 or more nucleotide bands which can be resolved by electrophoresis, thus permitting at least 80 different γ gene clonotypes (sets of rearrangements forming a single band) to be distinguished. We have also compared major T cell subsets in normal skin and normal gingival mucosa to attempt to rationalise T lymphocyte clonotype findings.

Four healthy adults (3M; 1F) provided samples of DNA from peripheral blood mononuclear cells (PBMC) and 6mm diameter punch biopsies of skin. Two of the subjects also supplied additional 4mm² biopsies of clinically healthy gingival mucosa. To establish whether specific clonotypes were associated with particular anatomical sites, 8 of the skin samples were obtained from symmetrical locations (e.g. right and left forearms, right and left chest) and the gingival specimens were taken from contralateral buccal midpoints of the upper right and upper left first molars. To permit comparison of CD3, CD4, CD8 and HECA

positive T lymphocyte subsets within normal skin and gingiva, frozen sections from the gingival and normal skin biopsies were obtained. DNA was isolated from the samples and then the products of 35 PCR cycles with a primer for Jγ1,2 paired with primers for Vγ2, Vγ4, Vγ8 and Vγ10 were electrophoresed on denaturing 6% polyacrylamide gels and autoradiography performed (8). Autoradiographic band patterns, as in Fig. 1, were scanned by laser densitometer (LKB, Sweden) and the absorbance values of the samples were then clustered by the method of unweighted pair group average linkage, to estimate similarities (10,11). 5μm serial cryostat sections of snap frozen tissue biopsies were immunohistochemically stained using mouse monoclonal antibodies specific for CD3, CD8, CD4 (SAPU, Scotland) and HECA 452 (a gift from CJLM Meijer, Amsterdam). Epithelial and connective tissue infiltrates were analysed as previously described (12,2).

With each primer combination studied, multiple clonotypes which vary from individual to individual were found in blood, skin and gingival mucosa. The similarities between tissues from contralateral sites within the same individual are illustrated by the Vγ10 gene rearrangements in Fig. 1. Band patterns differed between blood, skin and gingiva but tended to be qualitatively and quantitatively similar in multiple skin biopsies or in paired gingival samples from the same subject (Fig. 1). Findings similar to those seen in normal skin have been obtained in lesions of the T cell-associated cutaneous disorders psoriasis (13) and vitiligo (14). Immunohistochemistry shows consistently greater numbers of CD3, CD4, CD8 and HECA 452 positive T cells in the epithelium and connective tissue of the gingival mucosa than in epidermis and dermis.

Recent reports of a subset of circulating skin-homing memory T lymphocytes, which constitutively express the skin associated

T cell antigen defined by the monoclonal antibody HECA 452 and bind selectively to the endothelial adhesion molecule ELAM-1, suggest the existence of a corresponding subset of skin-homing T cell clones. We have thus investigated this possibility using highly sensitive PCRs which permit detection of clonal TCRγ gene rearrangements in 1-5 percent of a few hundred lymphocytes (8). T cell receptor γ (gamma) gene rearrangements were used as clonal markers of T cells in blood, skin and gingival mucosa from healthy human subjects. In each tissue there was marked clonal heterogeneity, as evidenced by the presence of multiple gene rearrangements (junctional diversity) of all four Vγ genes investigated. The striking similarity in the rearrangement profiles when skin was compared to skin or gingiva to gingiva indicate selective localisation of different subsets of T cell clones in skin and gingiva. These findings are consistent with the existence of local immune systems composed of skin-homing and gingiva-homing memory T cells. However the alternative possibility exists that specific T cells proliferate locally within certain tissues giving rise to characteristic T cell clones for these regions. In addition to clonal differences there are markedly more CD3, CD4, CD8 and HECA positive T lymphocytes in clinically 'healthy' gingiva, which may be related to the fact that ELAM-1 is strongly expressed in vascular endothelium of normal gingival mucosa (1). Other factors which might influence the localisation of dominant T cell clones in the gingival mucosa include expansion of specific T cell clones in response to local antigenic stimulation and upregulation of general adhesion molecules such as ICAM-1 (1) which may attract T cell subsets other than those which home to normal skin. Despite its importance, much remains to be learned about the structure and function of the skin immune system in health and disease (12).

This study shows that high resolution electrophoretic analysis of PCR products from Vγ and Jγ primers can provide new information about the anatomical distribution of T cell clonotypes and specifically that T cells localised to 'healthy' gingiva are remarkably similar between contralateral sites but differ from T cells of skin and peripheral blood. Continuing work may elucidate T cell clonotypes specific for patients with particular forms of periodontal disease.

REFERENCES

1. Kinane DF, Adonogianaki E, Moughal N, Winstanley FP, Mooney J, Thornhill M. Immunocytochemical characterisation of cellular infiltrate, related endothelial changes and determination of GCF acute phase proteins during human experimental gingivitis. *J Periodont Res* 1991; 26: 286-288.
2. Moughal NA, Adonogianaki E, Thornhill MH & Kinane DF. Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and Intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally induced gingivitis. *J Periodont Res* 1992; (in press).
3. Picker LJ, Michie SA, Rott LS, Butcher EC. A unique phenotype of skin-associated lymphocytes in humans. Preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. *Am J Pathol* 1990; 136: 1053-1068.
4. Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991; 349: 796-799.
5. Burnet M. *The Clonal Selection Theory of Acquired Immunity*. Cambridge: University Press, 1959: 156pp.
6. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature* 1988; 334: 395-402.
7. Rauiet DH. The structure, function and molecular genetics of the γ/delta T cell receptor. *Ann Rev Immunol* 1989; 7: 175-207.
8. Goudie RB, Karim SN, Mills K, Alcorn M, Lee FD. A sensitive method of screening for dominant T cell clones by amplification of T cell gamma gene rearrangements with the polymerase chain reaction. *J Pathol* 1990; 162: 191-196.
9. Goudie RB. A strategy for demonstrating the clonal origin of small numbers of T lymphocytes in histopathological specimens. *J Pathol* 1989; 158: 261-265.
10. Jackman PJH, Feltham RKA, Sneath PHA. A program in Basic for numerical taxonomy of micro-organisms based on electrophoretic protein patterns. *Microbiol Letters* 1983; 23: 87-98.
11. Kersters K. Numerical methods in the classification of bacteria by protein electrophoresis. In: Goodfellow M, Jones D, Priest FG

eds. *Computer Assisted Bacterial Systematics*. London: Academic Press, 1985; 337-368.
12. Bos JD, Zonneveld I, Das PK, Krieg SR, Kapsenberg ML. The skin immune system (SIS): distribution and immunophenotype of lymphocyte in normal human skin. *J Invest Dermatol* 1987; 88: 569-573.
13. Bos JD, Hulsebosch EJ, Krieg SR, Bakker DM, Gormane RH. Immunocompetent cells in psoriasis. In situ immunophenotyping by monoclonal antibodies. *Arch Dermatol Res* 1983; 275: 181-189.
14. Al Badri AMT, Todd P, Seywright M, Goudie RB. Dermal and epidermal lymphocytes in vitiligo. *J Pathol* 1990; 160: 163A.

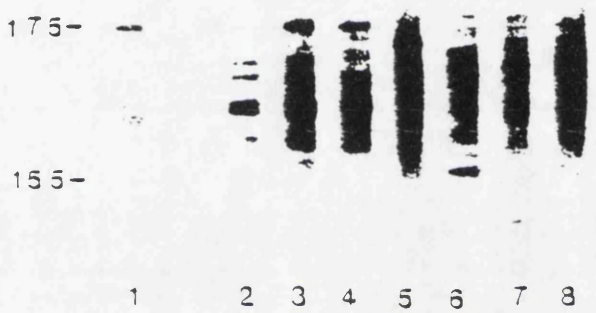

Figure 1. Comparison of T cell clones with Vγ10 - Jγ1,2 T cell receptor gene rearrangements in blood, skin and gingival mucosa of healthy subjects. Subject C (male 34y): lane 1, PBMC; lane 2; right arm; lanes 3 and 4, right and left gingiva. Subject D (female 30y): lane 5, PBMC; lane 6, right arm; lanes 7 and 8, right and left gingiva. Relative molecular mass calibration (in nucleotides) calculated from DNA molecular weight marker V. The range of molecular sizes is due to variation in rearrangement breakpoints and number of N-region nucleotides in different clones.