POLYMORPHONUCLEAR LEUCOCYTES AND PROTEASE INHIBITORS IN THE GINGIVAL CREVICE

By

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Abbreviations

ACJ:	Amelocemental junction
JE:	Junctional epithelium
PMN:	Polymorphonuclear leukocyte
LPS:	Lipopolysaccharide
IL-1:	Interleukin 1
GCF:	Gingival crevicular fluid
CT:	Connective tissue
IL-8:	Interleukin 8
ELISA:	Enzyme linked immunosorbent assay
BM:	Basement membrane
Ig:	Immunoglobulin
ul:	microlitres
IL-3:	Interleukin 3
IL-8:	Interleukin 8
CSF:	Colony stimulating factor
å:	Angstroms
ER:	Endoplasmic reticulum
ICAM 1:	Intracellular adhesion molecule 1
ICAM 2:	Intracellular adhesion molecule 2
VCAM 1:	Vascular cell adhesion molecule 1
LeuCAM:	Leukocyte cell adhesion molecule
LFA 1:	Lymphocyte function associated antigen
MAC 1:	CD11b-CD18 complex on PMN
ELAM 1:	Endothelial leukocyte adhesion molecule
TNF a:	Tumour necrosis factor alpha
GTPase:	Guanosine triphosphate phosphotase

GDP:	Guanosine diphosphate
GTP:	Guanosine triphosphate
ECM:	Extracellular matrix
LTB4:	Leukotriene B4
FMLP:	N-formylmethionyl-leucylphenylalanine
PAF:	Platelet activating factor
NADPH:	Reduced form of nicotinamide-adenine dinucleotide
O_2:	Oxygen
H^+:	Hydrogen
H_2O_2:	Hydrogen peroxide
OH^-:	Hydroxyl
H_2O:	Water
Fe:	Iron
B/PI:	Bactericidal/permeability increasing protein
CLCP:	Chymotrypsin like cationic protein
ARDS:	Adult respiratory distress syndrome
mg:	milligrams
ml:	millilitres
mm:	millimetres
PBS:	Phosphate buffered saline
°C:	Degrees centigrade
BSA:	Bovine serum albumin
OPD:	O-Phenylenediamide dihydrochloride
ul:	microlitres
M:	molar
IB:	Incubation buffer
HRP:	Horse radish peroxidase
CB:	Coating buffer
WB:	Wash buffer

nm:	nanometres
ng:	nanograms
SD:	Standard deviation
PVC:	Polyvinyl chloride

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There are so many people and situations that have influenced the starting, the slow progress and the eventual completion of this work that it's hard to know who to start with, but like everything it's best to start at the beginning.

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When I went out I stole an orange
I kept it in my pocket
It felt like a warm planet

Everywhere I went smelt of oranges
Whenever I got into an awkward situation
I'd take the orange out and smell it

And immediately on even dead branches I saw
The lovely and fierce orange blossom
That smells so much of joy

When I went out I stole an orange
It was a safeguard against imagining
there was nothing bright or special in the world

Brian Patten

DECLARATION

This thesis is the original work of the author

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SUMMARY

This thesis presents the results of a cross sectional clinical study of the PMN granule markers elastase and lactoferrin in conjunction with the major protease inhibitors alpha 1 antitrypsin and alpha 2 macroglobulin in the gingival crevice. 10 subjects with chronic adult periodontitis were selected. Each subject had GCF collected from a healthy site, a gingivitis site and a periodontitis site. The samples were analysed by ELISA. Analysis of the date confirmed that the sites were separated on the basis of clinical findings.

With regard to the PMN granule markers, elastase was not able to discriminate between periodontal sites on the basis of absolute amounts or concentration whereas statistically significant differences were observed in relation to the absolute amount of lactoferrin at healthy and periodontitis sites. The relationship between lactoferrin and elastase changed significantly when comparing healthy and gingivitis sites with periodontitis sites, where a 10-fold decrease in the relative release of elastase was noted. It is proposed that this relationship of PMN granule markers may be a useful indicator of periodontal breakdown. In addition it may indicate basic changes in the dynamics of PMN activity in the gingival crevice in relation to the disease state.

With regard to protease inhibitors, alpha 1 antitrypsin increased significantly in absolute amounts at sites of inflammation but remained unchanged in relation to its concentration. Alpha 2 macroglobulin increased at sites of inflammation in absolute amounts and was at highest concentration at sites of gingivitis. At sites of inflammation the amount of protease inhibitor relative to elastase increased. Thus, there was no evidence of a defective, host related protease inhibitor mechanism.

CHAPTER 1

INTRODUCTION

1.1   Definitions

The periodontium is the collective name for the tissues forming the attachment apparatus between tooth and bone. It comprises the four tissues:

- Radicular cementum

- Gingiva

- Alveolar bone

- Periodontal ligament

(Lindhe, 1989)

Together these tissues serve to anchor and suspend the teeth in the jaws. An important function of the gingival tissues is to form a physical barrier, mechanically sealing the interface between the oral cavity and the bone, thereby providing a primary defence mechanism against potential pathogens.

In relation to periodontal inflammation and the cells associated with acute inflammatory states, polymorphonuclear leucocytes, the anatomy of the gingival tissue is important. The gingival tissue covers the alveolar bone of the facial and lingual surfaces and encircles the teeth close to the amelocemental junction, where it is continuous with the junctional epithelium thus protecting the underlying tissue (Schroeder 1991).

Two zones are recognized macroscopically:

- Free gingivae - follows the scalloped contour of the necks of the teeth along the ACJ. The depth of the free gingiva is dependent on age and varies between 0.8-2.1mm in health.

- Attached gingivae - is bounded coronally by the marginal groove and apically by the muco-gingival margin. Again, its width is variable, being absent on the palate and widest

over the maxillary lateral incisors. In health it appears pale pink and has a stippled texture, similar to the surface of an orange. This stippling represents indentations of connective tissue into the epithelium.

The gingival sulcus, or crevice, is the groove between the gingival margin and the tooth. It is open occlusally, the lateral surface of the tooth forms its inner boundary, the coronal portion of the JE its apical boundary and the oral sulcular epithelium its lateral wall. It is a very dynamic region.

The oral sulcular epithelium is structurally like the oral gingival epithelium with a stratified squamous, parakeratinised epithelium. This layer is not permeable to PMNs.

Non keratinised JE forms a collar around the tooth on the cervical enamel or cementum. It extends from the cemento-enamel margin to the bottom of the gingival sulcus in health and is continuous coronally with the oral sulcular epithelium. The JE is derived from the reduced enamel epithelium and consists of 1-3 cell layers apically, widening to 15-30 layers coronally. It has 2 distinct strata; the basal layer, which lies on the basal lamina and is mitotically active, and the supra-basal layer. The interface between the connective tissue and the basal epithelium is normally smooth and has few intercellular connections. Importantly, there are no diffusion barriers across these cells. The JE tissue is constantly renewed. Cells originate at the basal layers adjacent to the connective tissue then move suprabasally towards the tooth and coronally along the basal lamina in the process of renewal. They are ultimately shed into the gingival sulcus. The JE is capable of regeneration and reattachment in 5-7 days. At the base of the gingival sulcus the cells of the junctional epithelium are exfoliated. Due to a lack of diffusion barriers and the nature of the intercellular connections of the JE it is permeable. PMNs migrate continuously from the vessels in the gingival connective tissue into the crevice. The number of PMNs migrating varies widely and is primarily dependent on the degree of tissue inflammation.

1.2   Periodontal Disease States

Periodontal diseases are inflammatory, bacterially induced conditions. They are recognized by a number of signs, dependent on the nature and severity of the disease. These include loss of periodontal attachment, bleeding on probing into the gingival sulcus, periodontal pocket formation, tooth mobility and tooth loss (Claffey 1994).

Periodontal disease, in one of its forms, has been reported to affect almost all of the population by adulthood (Marshall-Day et al 1955). It is advanced enough to pose a serious oral threat in a maximum of 10-20% of the population i.e. potentially to result in tooth loss (Papapanou 1994). Disease severity on a population level, as indicated by periodontal pocketing of increased depth and extent, increases with age (Yoneyama et al 1988).

On the basis of clinical findings periodontal tissues can be defined as :

- Healthy

- Superficially inflamed, with inflammation confined to the soft tissues and not involving apical migration of the epithelial attachment or bone loss i.e. Gingivitis

- Periodontally involved, in which there is apical migration of the junctional epithelium and loss of the periodontal attachment apparatus.

1.2.1 Gingival Health

This is a state characterised by the lack of clinical signs of inflammation. The gingival tissues appear firm and stippled and are pale pink in colour. They have a scalloped outline with the gingival margin following the line of the ACJ. Bleeding on probing in the gingival sulcus is

absent. The JE is attached to the coronal enamel and terminates at the ACJ. The alveolar bone crest lies within 2mm of the ACJ.

Histologically the tissue is characterised by small numbers of inflammatory cells in the junctional epithelium and immediately sub-jacent. These are principally PMNs but also include macrophages, monocytes, lymphocytes and plasma cells (Page and Schroeder 1976, Lindhe and Rylander 1975).

The bacteria associated with periodontal health are generally Gram positive cocci with few motile organisms, i.e. *Streptococcus sanguis, Streptococcus mitis, Actinomyces viscosus, Veillonella parvula, Actinomyces naeslundi, Staphylococcus epidermidis* (Socransky and Haffajee 1992).

1.2.2 Gingivitis

Gingivitis is inflammation of the gingivae. It is characterised clinically by oedema and redness of the marginal gingival tissues. There may be some loss of contour with interproximal swelling being present at an early stage. Bleeding on probing is present and the gingival crevice is deepened. It is present in most cases without underlying periodontal bone loss.

Gingivitis represents a tissue dynamic, whereby the host response confines the inflammatory process to superficial tissues. Irreversible tissue loss does not take place i.e. there is an effective host reaction which serves to limit and contain tissue damage (Attstrom and van der Velden 1994). The mechanism whereby this containment occurs is not yet understood. It is one of the major limiting factors in the successful use of markers to display periodontal activity as in only the minority of cases does gingivitis proceed to periodontitis.

1.2.3 Periodontitis

Periodontitis involves inflammation and destruction of the supporting tissues of the tooth and is essentially characterised by apical migration of the JE and loss of alveolar bone.

Clinical features are variable depending on the age of the patient and the severity of the condition. Signs and symptoms include bleeding on probing into the gingival crevice, an increased clinical probing depth or loss of attachment and radiographic evidence of periodontal bone loss. Gingivitis may or may not be present. Pain is not generally a feature of the disease, but is present in some conditions i.e. acute necrotising periodontitis. Recession, tooth mobility and drifting may be present.

1.3   Aetiology of Periodontitis

Following the demonstration of a clear correlation between plaque accumulation and the development of gingivitis in experimental trials (Loe et al 1965, Seymour et al 1983), plaque is acknowledged to be the major aetiological factor in periodontal diseases`(Kornman 1986). When plaque is allowed to accumulate on teeth or restorations at the gingival margin periodontal inflammation develops over a variable time span and with variable severity (Loe et al 1965, Socransky et al 1984).

Evidence for the existence of groups of individuals with higher susceptibility to the development of periodontal diseases has been presented (Johnson et al 1988). In their study of Sri Lankan tea workers Loe et al (1986) found 3 categories of subjects who could be identified; those with no attachment loss over the study period, those with moderate attachment loss and those with rapid attachment loss which resulted in tooth loss over the study period. This principle, of a range of periodontal susceptibilities, has been confirmed

in a UK population. Cushing and Sheiham (1983) found deep pockets in only 7% of a British population. Some of this susceptibility may be genetically determined or related (Wilton et al 1988, Sofaer 1990).

The microbiology of plaques associated with periodontal diseases has been studied extensively (Moore 1987, Maiden et al 1990). Up to 400 species have been isolated from plaque samples and identified in association with disease or health (Socransky and Haffajee 1992). The obvious problem is to discriminate between those organisms which are associated with disease and those which cause it. Socransky and Haffajee (1991,1992) discussed the important criteria for organisms in relation to periodontal diseases. They proposed that pathogens should have the following characteristics :

- be of a virulent clonal type

- possess the chromosomal and extra-chromosomal genetic factors to initiate disease

- that the host should be susceptible to the organism

- that the pathogen should be present in such numbers as to exceed the host threshold to protect itself

- that the pathogen should be located in the correct place

- that other bacteria must foster, or at least not inhibit, the process

- that the local environment must be one which is conducive to the expression of the species virulence properties

The principal concept now emerging in microbiological terms is one of bacterial ecology rather than specific infectious agents i.e. pathogenic plaques in the absence of protective species (Newman 1990). The bacteria most often implicated in periodontitis are *Actinobacillus actinomycetemcomitans* (Zambon 1985), *Porphyromonas gingivalis, Prevotella intermedia* (van Winkelhoff et al 1988) and spirochetes (Loesche and Laughon

1982). For some organisms e.g. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*, a carrier status at healthy sites is reported. The overgrowth of these oral commensals above the level of the host resistance may be what leads to periodontitis (Theilade 1986). Preus et al (1987), reported cases of early onset periodontal disease in which sites with active bone loss harboured *Actinobacillus actinomycetemcomitans* with bacteriophage infection whereas those without bacteriophage infections had no active bone loss. Although the data they presented was anecdotal, they proposed this as a possible mechanism whereby commensal organisms become pathogenic.

Birkedel-Hansen (1993) proposed that bacterially induced damage is due partly to the release of proteolytic enzymes which destroy tissues directly. Bacterial products may also suppress local inflammatory responses e.g. leukotoxin production by *Actinobacillus actinomycetemcomitans* (Baehni et al 1979, Slots and Dahlen 1985). In addition to this independently mediated bacterial action bacteria produce toxins, LPS or enzymes which trigger macrophages, fibroblasts, keratinocytes and endothelial cells. The final pathway of tissue destruction in periodontitis is dependent not only on direct bacterial action but also on the release from triggered host cells of inflammatory mediators (Slots and Genco 1984).

Direct tissue invasion associated with diseased tissue has been demonstrated in acute necrotising ulcerative gingivitis (Listgarten 1965, Loesche et al 1982). Other reports of tissue invasion have been questioned as artefactual, being caused by sampling techniques which introduce bacteria. Recently the ability of bacteria to invade cells in culture has been demonstrated (Sandros et al 1993, 1994). This capacity to invade tissue has been associated with clinical difficulties in the elimination of bacteria and treatment of disease (Saglie et al 1981).

The host has several superficial protective mechanisms (Genco and Slots 1984). These include; prevention of bacterial colonisation, the integrity of the junctional epithelium as a

physical barrier, the flow of GCF and desquamation of cells and the release of toxic or irritant substances by bacteria in the crevice.

Beyond the superficial defence the provocation of local inflammatory and immune responses occurs. The inflammatory reaction is mounted initially by PMNs in the gingival crevice. Examination of individuals with abnormal PMN responses, either qualitatively or quantitatively, shows that these individuals have early onset or rapidly progressing periodontal breakdown compared with those without such defects (Wilton et al 1988). In view of the poor outcome if PMNs are not functioning the potentially damaging effects of these cells is accepted as collateral damage and the cells appear to be of clinical benefit. Other facets of the host response rely on the actions of T cells in cell mediated immunity, B cells and plasma cells in antibody production and monocytes and macrophages in the elimination of bacteria (Ranney 1991).

Recent evidence regarding the effect of cigarette smoking has shown this is of major importance in terms of susceptibility to disease and difficulties in its treatment (Haber et al 1993, Bergstrom 1994). Smokers have been shown to have more bone loss (Arno et al 1959) than non smokers, a poorer response to treatments, both surgically and non surgically (Preber and Bergstrom 1985, 1990), with this difference being more marked when sophisticated treatment is provided. The mechanism for this susceptibility to disease and resistance to treatment is not understood, but is thought to have both local and systemic components and to be unrelated to plaque (Bergstrom et al 1991). In lung disease cigarette smoking is proposed to have a role in the oxidation of alpha 1 antitrypsin, disabling it and reducing the protective screen against elastase in the alveolar tissue (Gadek et al 1981); Alavi et al (1995) proposed a similar mechanism in the periodontium.

Local factors e.g. crowded teeth, mouth breathing have been proposed as of importance in the aetiology of periodontal disease. These are now thought to contribute to damage by the promotion of plaque accumulation but not by altering the host susceptibility.

Despite knowledge of the aetiology of periodontal tissue loss there still exists no reliable method for the prediction or immediate detection of clinical disease. Several theories regarding the temporal nature of tissue loss have been proposed. Epidemiological data supports the concept that periodontal attachment loss is progressive with age (Papapanou 1994), this was proposed as the slow continuous pattern (Socransky et al 1984). Socransky et al (1984) disputed the validity of this model on the basis of the clinical findings when monitoring longitudinal attachment loss. They suggested that attachment level changes were not continuous but rapid and of short duration, in addition that they were random in distribution. The development of their alternative model came about largely because of the difficulties involved in longitudinal monitoring of attachment loss and the probing differences required to reliably diagnose that this had occurred. With the more widespread use of the Florida probe the threshold for recognising changes in attachment level is reduced and the concept of random quick periods of attachment loss is being disputed. Despite improvements in the reliability of periodontal probing, the search is still underway for a clinically useful test to diagnose periodontitis before irreversible tissue damage occurs.

1.4   Gingival Crevicular Fluid

Gingival crevicular fluid is fluid which can be recovered from the gingival crevice. It is derived principally from serum and contains cells, bacteria and tissue products. It has been estimated that between 0.5 and 2.4 ml of GCF flows into the oral cavity per day (Cimasoni 1983).

Several theories of formation for GCF have been proposed. Brill and Krasse (1958), Brill and Bjorn (1959) and Egelberg (1966), related GCF production to inflammatory changes in the connective tissue vessels underlying the JE resulting in leakage of serum. They suggested that the permeability of JE regulated fluid flow outwards into the gingival crevice to form the GCF.

Alfano (1974), proposed that the flow of GCF was due to the effects of osmotically active substances in the gingival crevice e.g. plaque products. Under normal circumstances these products, in small amounts, would be removed by absorption onto desquamating epithelial cells and would have a small effect. At sites of plaque accumulation, the normal turnover of epithelial cells forming this local defence would be insufficient to control the accumulation of osmotically active products. Thus, macromolecules could diffuse intercellularly and accumulate at the basement membrane establishing an osmotic gradient between the crevice and the connective tissue. This gradient would promote the flow of fluid from the connective tissue. The innate permeability of the junctional epithelium is again of crucial importance in this theory.

Pashley (1976), described a model for GCF production which postulated that GCF production was a balance between passage of fluid from capillaries into tissue and its subsequent uptake by lymphatic vessels. If the balance is altered, increasing the filtration rate from capillaries to a level above that of the lymphatic drainage rate, fluid accumulates in the interstitial fluid compartment, crosses the basement membrane and JE and passes into the gingival crevice.

GCF contains cells, electrolytes, organic compounds including carbohydrates, immunoglobulins, complement, lipids, cell metabolites, bacterial products and enzymes (Cimasoni 1983). Of most interest in the present study is the cellular content.

Loe (1961) reported the cellular component of GCF to be epithelial cells, shed from the JE, and leukocytes. Attstrom and Egelberg (1970) reported the differential leukocyte count in the gingival crevice as PMNs 95-97%, lymphocytes 1-2%, monocytes 2-3%. By far the most prominent cell type in GCF is the PMN. When recovered approximately 80% of PMNs are viable (Skapski and Lehner 1976) and possess residual phagocytic capacity (Kowolik and Raeburn 1980, Thurre et al 1984). The numbers of white cells emigrating from blood vessels through the junctional epithelium and into the sulcus increases with the degree of inflammation in the gingival tissues (Attstrom 1970, Loe and Silness 1963, Adonogianaki et al 1993, Kowashi 1980, Thurre et al 1984, Schiott and Loe 1970). *In vitro* and *in vivo* studies have demonstrated the ability of supragingival plaque to increase PMN migration, increase vascular permeability in the dentogingival vessels and increase GCF flow (Hellden and Lindhe 1973) i.e. to be chemotactic.

The majority of GCF is plasma derived with local conditions altering the rate at which fluid leaves capillaries. The permeability of the junctional epithelium enables the passage of fluid, cells and cellular products into the gingival crevice. What is contained in the GCF eventually entering the crevice varies according to the local conditions, thus is of interest as a potential source of disease markers.

1.4.1 Collection of GCF

Several methods of collection of GCF have been described. The various methods to consider are:

- Use of absorbent paper strips

- sampling with micropipettes

- collection of gingival washings

Each of these methods have slight differences in technique which makes their application suited for some investigations rather than others. The present study employed the use of absorbent paper strips and this technique will be discussed further.

Brill and Krasse (1958) described collection of GCF using absorbent strips. In the extra crevicular method strips are placed over the orifice of the gingival crevice on the vestibular surface of the teeth, marginal and attached gingivae. This method avoids all trauma to sulcular and junctional epithelium during collection but involves the risk of salivary contamination of the strip. In contrast, intra crevicular sampling involves placing the filter strip into the gingival crevice until resistance is felt. The strip is placed atraumatically to avoid contamination.

Griffiths et al (1988) compared filter strips and assessed their suitability with regard to GCF collection and compatibility with the Peritron 6000, used in the estimation of GCF volume. They found the absorbency of Whatmann 3MM chromatography papers allowed high uptake of fluid prior to saturation when compared with Periopapers. In addition they showed good recovery of serum proteins following elution over a wide volume range from these strips.

Nakashima et al (1994) compared the recovery of PMNs and enzymes from Periopaper, Whatmann 3MM chromatography paper, paper points and Durapore filter membranes. All materials except paper points released more than 90% of sampled enzymes into saline and were considered satisfactory for the recovery of GCF enzymes.

Durapore filters have been shown to be useful for the recovery of intact PMNs from the gingival crevice (Andersen and Cimasoni 1993). They reported a recovery rate of around 90%, with cells being easily removed from the Durapore during elution compared with other

strips tested. They proposed that collection of GCF using Durapore filters was valuable for direct evaluation of PMN number and enzyme levels in the same sample.

The length of sampling time affects the composition of GCF and times varying between 5 seconds and 3 minutes have been suggested (Loe and Holm-Pedersen 1965, Biswas et al 1977, Hattingh and Ho 1980, Novaes et al 1980, Curtis et al 1988). The most commonly employed sampling time is 30 seconds. This appears to cause little trauma or alteration in the dynamics of the crevice while enabling collection of adequate quantities of GCF for examination (Lamster et al 1986).

Quantification of the volume of fluid collected from the crevice has been carried out by weighing of the strip (Valazza et al 1972), estimation of the amount of wetting of the strip (Golub et al 1971, Egelberg and Attstrom 1973) and now commonly with the Periotron. The Periotron is an instrument which measures the electrical flow between its upper and lower jaws when a moistened paper strip is placed between them. The flow is a reflection of the degree of wetting of the paper. This can be calibrated against known volumes of fluid and the appropriate conversion made to give the volume of GCF. When used consistently, with standardisation of strip placement and daily calibration this method has proven useful and reliable (Hinrichs et al 1984, Stewart and Maeder 1986, Stewart et al 1993). Recently, however, Chapelle et al (1995) reported that at sample volumes below 0.2ul the reliability of the Periotron 6000 was greatly reduced. The differences in fluid volume readings between machines was significant. In addition to the unreliability of the Periotron with small volumes, problems occur due to evaporation from strips (Curtis et al 1988). When small and dissimilar sample volumes are being compared, this has a proportionately large effect on volume measurement and may be significant when calculating concentrations of constituents in GCF (Lamster et al 1986, Imrey 1986). These findings further supports the reporting of absolute amounts of GCF constituents rather than concentrations in clinical trials.

1.5   GCF as a source of periodontal markers

The clinical difficulties in identifying periods of disease activity has led to the search for markers of disease which are sensitive to changes in the local environment or which precede the clinical changes. Due to the contribution of locally derived components to GCF it has been proposed as a major source of this information (Fine and Mandel 1986, Curtis 1989 et al, Harper et al 1989, Lamster 1991, Lamster 1992, Offenbacher et al 1993, Lamster et al 1994, McCulloch 1994). Proposed markers are related to aspects of the pathogenesis of the disease as outlined above. Curtis et al (1989) defined the potentially useful components of GCF:

- Products derived from microbial plaque e.g. endotoxin, enzymes and end products

- Products of tissue breakdown e.g. collagens, proteoglycans, hyaluronic acid and other CT components

- Products of host cells e.g. desquamating epithelial cells and leukocytes, enzymes such as collagenase, elastase, cathepsin D, lysozyme, hyaluronidase, B-glucuronidase, aryl sulphatase and lactoferrin.

- Products of host immunity e.g. immunoglobulins, complement, IL1 and IL8, and arachidonic acid metabolites.

Modern laboratory methods have made identification of indicators much easier, especially where only small quantities of the component being examined exist. The number and range of substances which can be identified in GCF has increased greatly and commercial kits are now available. These kits rely on the quantification of one aspect of the pathogenesis of the disease process e.g. microbiology or cellular processes. However, they discriminate between inflammation and no inflammation, but not between the superficial inflammation in gingivitis and inflammation associated with the destruction in periodontitis. Recently the concept of a

risk profile, based on the incorporation of several markers within GCF has been developed (Lamster 1994 et al). Grbic et al (1995) combined immunoglobulin profiles and a PMN marker in GCF and suggested that such a profile was useful in the investigation of the host response. The developement of marker profiles may prove more useful than the use of single indicators in isolation. It is on the basis of the developement of a GCF profile of PMN degranulation at different categories of sites that the present study is based.

1.6   Polymorphonuclear leukocytes

The polymorphonuclear leukocyte is a phagocytic cell and is the predominant leucocyte in blood and in the gingival crevice (Attstrom & Egelberg 1970, Wilton 1986 and Bedeck Spat et al 1991). It forms about 62% of peripheral white blood cells and about 91% of gingival crevicular cells recovered by aspiration (Skapski & Lehner 1976). PMNs are found at sites of both gingival health and disease (Sharry & Krasse 1960, Attstrom & Egelberg 1970). The PMN is an important cell because, along with the integrity of the physical surface barrier, it forms the first line defence against invading pathogens.

PMNs develop from pluripotential bone marrow stem cells (Bainton et al 1971) and the cells go through several stages prior to maturity. The lifespan of a PMN outwith bone marrow is short, with an average of 7 (McGee et al 1992) to 58 (Bainton et al 1971) hours reported. The production of PMNs and their release from the bone marrow is closely controlled by colony stimulating factors, each of which controls a specific stage of maturation, amongst these are Interleukin 3, the complement fragments C3e and C3d.g, Granulocyte and Macrophage CSF, Interleukin 6, Granulocyte CSF, Monocyte CSF and Erythropoitin (Kanwar and Cairo 1993).

As PMNs mature they become more deformable, alter their surface charge and acquire new cell surface receptors (Wallace et al 1987). Following these changes mature PMNs can cross endothelial barriers in the bone marrow and enter the bloodstream.

In the circulation PMNs form two pools; circulating and marginated (Athens 1981). Marginated PMNs are 'stored' in the small vessels, particularly of the lung and spleen. They can be mobilised at short notice in times of acute need. The average range of PMN numbers in the circulation is 2.5-7.5 $\times 10^9$ cells/litre. The number can increase 10 fold within 28 hours in times of acute inflammation. This rapid and substantial increase in PMN number occurs as a result of several processes; transfer of cells from the marginated to the circulating pool, increased release of mature cells from the bone marrow and an increase in the rate of maturation of cells within the bone marrow. Numerous surface receptors are present on PMNs (Rosales and Brown 1993). Four major classes are identified; receptors for inflammatory mediators and bacterial products, receptors for lymphokines and monokines, opsonic receptors and receptors for endothelium and proteins of tissue matrix. These receptors are used for all stages of PMN emigration from blood vessels, chemotactic movement, recognition of foreign materials, its binding and phagocytosis and for the control of these processes via lymphokines and monokines.

The PMN has a characteristic light microscopic appearance with a multilobed nucleus and numerous cytoplasmic granules. It has a diameter of 12-15å. The granular component of the cell consists of many cytoplasmic granules with an outer membrane enclosing the densely packed protein in a mucopolysaccharide matrix.

Three types of granules exist :-

- primary or azurophil granules (Bainton et al 1971)

- secondary or specific granules (Bainton et al 1971)

- tertiary or C particles

The tertiary granule is not well characterised and is thought to contain gelatinase (Miyasaki et al 1994). Granule contents have been investigated by many workers (Baggiolini et al 1978, Spitznagel et al 1974, Bainton et al 1971, Falloon & Gallin 1986).

1.6.1   Primary Granules

Primary granules are first formed, at the promyelocytic stage, and develop within the Golgi apparatus and rough ER. They are identified by their peroxidase content. Positive staining for peroxidase is seen in the granules, at all stages, and in the PMNs secretory apparatus during their formation. The degree of peroxidase compaction is variable. Their appearance is not uniform, granules are either round or shaped like a rugby ball, with round granules predominating. Primary granules contain (Baynes and Bezwoda 1994):

Antimicrobial Agents   Defensins

                              Myeloperoxidase

                              Lysozyme

                              Cationic proteins

Proteinases   Elastase

                              Cathepsin G

The contents of the primary granules are largely responsible for the non oxidative killing mechanisms of the neutrophil (Miyasaki et al 1994)

1.6.2 Secondary granules

Secondary granules are formed at the myelocyte stage of PMN developement, after primary granules. They are peroxidase negative, of low density and are twice as numerous as primary granules in the mature PMN. Their size is variable and they are typically spheres or rods. All secondary granules have a similar content despite their morphological differences. Secondary granules contain (Baynes and Bezwoda 1994):

Antimicrobial agents	Lysozyme
Proteinases	Collagenase
Other	Lactoferrin
	B12 binding protein
	Laminin receptor(67K)
	CD11b/CD18 (C3bi)
	Fibronectin receptor alpha
	Vitronectin receptor alpha

The release characteristics and kinetics of primary and secondary granules are different. Secondary granules are released chiefly extracellularly, with the granules being found close to the advancing edge of cells responding to a chemotactic stimulus (Wright and Gallin 1979). Evidence of secondary granule release extracellurlarly is more prominent than that of primary granule release (Henson 1971) and occurs at an earlier stage (Bentwood and Henson 1980, Gustafsson et al 1994). These phenomenon have been interpreted in the past as indicating a secondary and extracellular function of secondary granules and an intracellular function of primary granules related to phagocytosis (Wright and Gallin 1979).

The process of degranulation is calcium dependent and is not related to cell death (Henson 1971, Wright and Gallin 1979 and Taichmann et al 1977) with lysozymal enzymes released without a rise in lactate dehydrogenase, an indicator of cell death. *In vitro*, granule release can be stimulated by dental plaque (Taichman et al 1977), binding of immune complexes and complement (Henson 1971(a)), binding to non phagocytosable surfaces (Henson 1971(b)) and passage through membranes (Wright and Gallin 1979).

1.7   Polymorphonuclear Leukocytes in Inflammation

When functioning efficiently against infectious agents PMNs leave blood vessels and move across tissues towards a chemotactic source where they attempt to kill the organism.

Adhesion molecules related to the movement of PMNs from vessels are expressed on endothelial cells and PMNs (Rosales and Brown 1993). During inflammation the molecules which are important for the binding of PMNs appear before those for other leukocytes, thus PMNs are the first cells to cross the vessel wall. The immediate adhesion of PMNs is mediated via upregulation of P-selectin on endothelial cells by thrombin or histamine (Patel et al 1991). Stimulation of post capillary venules by inflammatory agents or bacterial markers, including IL-1 and TNF, stimulates protein synthesis, upregulating the expression of the endothelial cell markers (Diamond 1991, Staunton et al 1990). This functions over the longer term i.e. 24 hours and increases the interaction of leukocytes with the cells of the vessel walls. The endothelial markers ICAM-1, ICAM-2 and VCAM-1 bind to the LeuCAM family of integrins which are expressed on PMNs. LFA-1 binds specifically to ICAM-1 and ICAM-2. MAC-1 also binds ICAM-1.

In response to the expression of surface markers the cells change shape and roll along the blood vessel wall. They eventually stop moving, become flat and their membranes develop a ruffled border. They then migrate through the endothelial wall. ELAM-1, which is necessary for this migration, is expressed in response to IL-1 and TNF. IL-1 and TNF-a thus have a crucial role in the control of inflammatory mechanisms following the initial stimulus (Page 1991).

PMNs are capable of responding to many different chemoattractants including N-formyl peptides, complement derived C5a, Leukotriene B4, interleukin 8 and platelet activating factor (Havarth 1991). Chemoattractant receptors span the cell membrane and are GTPase-coupled receptors. The intermediary G proteins of these receptors couple cell surface binding of the chemoattractant to the final effector enzyme systems of the PMN. The rate of conversion of GDP to GTP in the receptors serves to control the processes started by the initial cell surface binding of ligand.

Once outside the vessel, cells migrate through the connective tissue towards the chemotactic stimulus. Directed movement of PMNs comes about by the interaction of specific chemoattractants with surface receptors on its plasma membrane. Binding activates a cascade of secondary intercellular events which lead to either chemotaxis or the respiratory burst, dependent on the chemotactic agent (McPhail and Harvath 1993).

Neutrophils stimulated by chemoattractants undergo morphological changes becoming elongated cells with a ruffled border. During movement the anterior edge extends forwards and the posterior edge retracts towards the body of the cell (Cassimeris and Zigmond 1990). When attached to a substratum their advancing edge is directed towards the chemotactic stimulus and the contractile end posteriorly. Adherence of the cells to a substratum occurs via the glycoproteins and proteoglycans of the ECM. The concentration of ECM glycoproteins and proteoglycans changes at sites of gingival

inflammation (Bartold 1992, Purvis et al 1984) and these molecules can become chemotactic for inflammatory cells. Actual cell movement occurs by the polymerisation of globular actin to fibrillar actin and current models propose that the fibrillar actin is involved in the generation of the force required for cell movement (Onman et al 1987).

Chemoattractant receptors exist in two interconvertible forms; high and low affinity states (Sklar and Onman 1990). At high affinity sites the ligand slowly dissociates from the receptor whereas at low affinity sites dissociation is rapid. Chemotaxis is initiated at receptors by concentrations of chemoattractants which are much lower than those required to produce the respiratory burst. Snyderman and Pike (1984) proposed that binding of chemoattractant to high affinity sites results in chemotaxis whereas binding to low affinity sites results in degranulation. Kermode et al (1991) proposed that binding to a receptor converts it to a high affinity state which then results in degranulation. If the ligand has a high binding affinity or is extremely stable then binding is maintained. The maintenance of this state is necessary for chemotaxis. If the ligand is of low potency then the high affinity state is not sustained and chemotaxis does not take place. This difference in the effect of chemoattractants is known as chemoatracttant divergence (Havarth 1991). It has been suggested that endogenously generated chemoattractants recruit neutrophils to inflammatory sites with minimal activation of the respiratory burst. This provides a mechanism which maintains tissue homeostasis while preventing damage to the host tissue during neutrophil migration. Chemoattractants generated by invading organisms are, in contrast, more potent stimuli of both chemotaxis and oxidative metabolism in the PMN, as occurs in an infectious environment *in vivo*. Chemoattractants appear to be most effective in producing chemotaxis in the following order of potency; IL-8, LTB4, C5a, FMLP, PAF. For the production of the respiratory burst the following order of potency applies; FMLP, C5a, with PAF, IL8 and LTB4 all of similar effectiveness (McPhail and Havarth 1993).

Once at the site of the chemotactic stimulus and prior to attachment of the PMN to the invading organism, opsonisation takes place. Opsonisation can take place either in the presence or absence of complement and antibody.

In the presence of complement and antibody, activated C3 and IgG bind their respective PMN receptors, CR1 and CR3 for complement and FcR11 and FcR111 for antibody.

Capsular organisms are able to evade this opsonisation and phagocytosis due to the masking of the antigenic portion of their cell wall by the capsule and by avoiding the deposition of complement on their surface (Finlay and Falkow 1989, Slots and Genco 1984, Arnold 1993).

If antibody or complement are not available then alternative systems exist for the opsonisation of bacteria. Generally, cell wall antigens determine the ease with which an organism is phagocytosed. Lipopolysaccharide binding protein is an acute phase reactant which binds LPS. LPS is a cell wall component of gram negative bacteria. The binding of LPS binding protein to bacteria enables binding to the CD14 receptor on PMNs and macrophages thus enhancing phagocytosis (Wright et al 1989). Since gram negative bacteria are abundant in the gingival crevice and complement and antibody can be locally destroyed (Genco and Slots 1984) it is likely that this system is in operation.

Following opsonisation phagocytosis can occur. Phagocytosis is the process by which the PMN takes up a particle. It involves attachment of the phagocyte to the particle and its subsequent engulfment. It starts with the receptor-ligand binding between PMN and the microbe. This interaction activates the ingestion phases which involve actin, myosin and actin binding proteins. Actin microfilaments in the portion of the cytoplasm underlying the site of particle attachment undergo polymerization. This polymerization leads to

puckering of the plasma membrane at the site of contact where microfilaments are attached. The membrane surrounds the particle, pseudopodia are produced and a phagocytic vacuole is formed. Simultaneously cytoplasmic granules fuse with the phagosome membrane to form a phagolysosome. This final fusion of the membranes and release of granule contents generally results in destruction of the engulfed particle (Verhoef and Visser 1993).

Once phagocytosed and engulfed by the PMN 2 mechanisms exist to kill the organism; oxygen dependent and oxygen independent mechanisms (Van Dyke et al 1985).

Oxygen dependent killing is activated when the PMN undergoes a respiratory burst coincident with phagocytosis. NADPH in the phagolysosome membrane is activated and reduces O_2 to superoxide (Weiss 1989).

The proposed sequence of activation of the NADPH oxidase complex is illustrated below:

1) $2 O_2 + NADPH \text{ -------------} > 2 O_2^- + NADP^+ + H^+$

NADPH oxidase

2) $2 O_2^- + 2 H^+ \text{ -------------} > H_2O_2 + O_2$
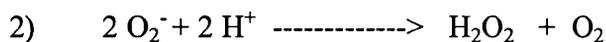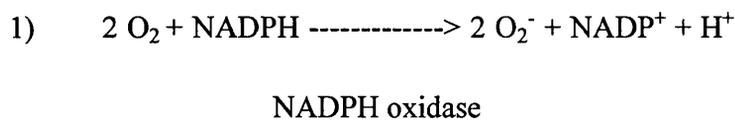
3) The Haber Weiss reaction:

$O_{2-} + H_2O_2 \text{ ------------} > OH^- + OH + O_2$
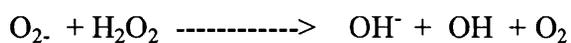
The third step in this sequence is often discussed but has never been demonstrated *in vivo*. Following the generation of hydrogen peroxide and OH in this reaction it is proposed that a second enzyme system involving myelperoxidase, a primary granule component, becomes active. Myelperoxidase is released by PMNs in large quantities but on its own has little effect. In combination with H_2O_2, myeloperoxidase is capable of catalysing the oxidation of plasma halides, the most important of which is chloride because of its concentration.

$$H_2O_2 + Halide + H^+ \text{-------------} > HOX + H_2O$$

(X)   myelperoxidase (halide)

It is at this stage that the interaction between chlorinated oxidants, protease inhibitors and proteases can occur. Alpha 1 antitrypsin contains a methionine residue which is sensitive to oxidation, resulting in a 2000 fold decrease in its association with elastase. Weiss (1989) proposed that chlorinated oxidants produced as a result of the NADPH/myeloperoxidase reactions, oxidate the protease inhibitor, thereby creating a zone in which extracellularly released elastase can directly act on its substrate. Once bound to its substrate, elastase is insensitive to further inhibition by protease inhibitors. This mechanism was also proposed in relation the other major protease inhibitor alpha 2 macroglobulin. The importance of this system in the immobilisation of protease inhibitors *in vivo* is however now questioned.

In vivo, in the presence of iron, H_2O_2 and superoxide, an alternative to the above Haber-Weiss reaction, the Fenton reaction, is proposed (Morris et al 1995). OH production from H_2O_2 and O_2 is dependent on iron availability, with iron being reduced to Fe_2^+ during the reaction. OH is considered the most toxic of all the free radicals. If this reaction occurs *in vivo* then the rate limiting step is the availability of iron. Little free iron is available due to its binding to transferrin and lactoferrin extracellularly and apoferritin intracellularly

(Sanchez et al 1992). In the phagocytic vacuole the pH is reduced and some iron may be lost from transferrin, however, lactoferrin retains its ability to bind iron at pH4 thus protecting the cell and limiting the production of OH (Halliwell and Gutteridge 1985).

Oxygen independent killing is based on the PMN granule network. Antimicrobial agents which are independent of O_2 are released into the phagolysosome during phagocytosis (Thomas et al 1988). These granule components, including bactericidal/permeability-increasing protein, chymotrypsin-like cationic protein and defensins (Weiss et al 1982, Verhoef and Visser 1993) and those outlined above, are strongly bacteriocidal and in most cases effectively kill bacteria (Miyasaki et al 1994). In addition to killing bacteria the proteolytic enzymes, including elastase and the metalloproteinases have the potential to damage extracellular tissue and have been suggested to operate in periodontal tissues (Ohlsson et al 1974, Sandholm 1986, Birkedal Hansen 1993,1994)

1.8   Granule Components

1.8.1   Elastase

Elastase is a serine protease, a member of the family of neutral proteases which includes Cathepsin G and collagenase and it is found in and released from primary granules. It was identified by Janoff and Schrerer (1968) and has a molecular weight of 27-31,000 with variable carbohydrate content. The mRNA for elastase is found in PMNs only up to the promyelocyte stage, following which production of the components of the primary granule ceases (Bainton et al 1971). Elastase is not present in all primary granules, but is found in a ratio of 400+ve per 1500 granules. PMNs not containing elastase are not morphologically different, but can be identified by density gradient centrifugation.

Elastase has many potential actions, reviewed and discussed by Woesner (1992), Cergeneux et al (1982), Ohlsson & Olsson (1974). *In vitro* these properties include cleavage of elastin, which has been demonstrated for elastase in GCF; degradation of types I, III and IV collagen, all of which are present in basement membrane and periodontal tissue; proteoglycan cleavage; fibronectin cleavage; degradation of complement factors, IgG, kininogen and clotting factors VIII, XII and XIII. *In vivo*, the functions of elastase are less clear.

The *in vivo* action of elastase is closely related to its binding with protease inhibitors (Ohlsson and Olsson 1974, Weiss 1989, Lamster 1991, Woessener 1992, Giannopolou et al 1992). The major serum protease inhibitors are alpha 1 antitrypsin, alpha 2 macroglobulin and secretory leukoproteinase inhibitor. Alpha 1 antitrypsin and alpha 2 macroglobulin have been identified in GCF (Ohlsson et al 1974)

Elastase is important in several disease processes (Leff and Repine 1993), particularly adult respiratory distress syndrome (ARDS). ARDS is a noncardiogenic, pulmonary oedema that occurs as a result of non specific damage to the alveolar capillary membrane. As a result of the damage, alveolar spaces are flooded with inflammatory cells. Bronchial lavage studies in patients with this condition have shown a large increase in levels of neutrophil products, notably elastase (Gadek et al 1981). Lieberman (1976) suggested that the damage in ARDS is brought about by an imbalance of the protease-antiprotease shield in the lung. Wewers et al (1988) demonstrated that the neutrophil elastase activity in ARDS lavage fluid was produced by elastase complexed to alpha 2 macroglobulin. They further suggested that although the complex was active against low molecular weight substrates, it was unlikely to be involved in tissue damage *in vivo*, but rather to function in immunomodulation.

In GCF elastase has been studied as a marker of diseases in two directions; the protease activity of elastase-like peptides and antigenic elastase content. Potentially these are two very different features of elastase (Cox 1995).

Investigation of protease-like activity may be of potential use as an indicator of tissue destruction since elastase is able, *in vitro*, to degrade some of the proteins of the periodontal tissues. The importance of that particular GCF protease is however difficult to determine. In addition, the *in vivo* action of elastase in GCF may not correspond to the *in vitro* action of the same amount of protease.

The alternative method, used in this study of elastase, is the direct measurement of the amount of elastase present, not its activity. The potential benefit of this is in understanding PMN dynamics in the inflammatory process. This is complicated by the fact that elastase exists not as an isolated enzyme but in combination with its inhibitors (Giannopolou et al 1992, Ohlsson and Olsson 1974).

It remains to be established whether in the gingival crevice elastase has the capacity to degrade connective tissue and disrupt basal lamina as has been described *in vitro*. Furthermore if it has that capacity whether the amounts that are found in GCF are sufficient to contribute directly to tissue damage or whether the importance of elastase and elastase like activity is in the disruption of other functions.

1.8.2 Lactoferrin

Lactoferrin is an iron binding protein found in the secondary granule of the PMN (Spitznagel et al 1974). In addition to chelating iron it is able to bind copper, but to a lesser extent. It is a member of the transferrin family.

Lactoferrin is believed to function against bacteria in several ways (reviewed by Ellison 1994); it is bacteriostatic, depriving bacteria of vital metallic growth factors (Masson & Hereman 1968), indirectly, by chelating metal ions it decreases the production of free radicals in the Haber Weiss reaction, described above (Britigan and Edeker 1991). The antimicrobial activity of IgG, IgM and IgA is increased in combination with lactoferrin (Duncan and McArthur 1981). In addition lactoferrin directly modifies the wall of Gram negative bacteria causing the release of LPS (Ellison 1988).

In relation to phagocytic cells and inflammation lactoferrin alters PMN adhesiveness and chemotaxis (Oseas et al 1981, Falloon & Gallin 1986). Britigan et al (1994) discussed the role of lactoferrin as an anti inflammatory agent. In addition to potentially limiting the production of hydroxyl radicals they suggested that lactoferrin binding of LPS altered its bioavailability thus reducing the inflammatory reaction.

1.9   Protease Inhibitors

The two major serum protease inhibitors, alpha 1 antitrypsin and alpha 2 macroglobulin are present in GCF (Ohlsson et al 1974).

1.9.1   Alpha 1 anti trypsin

Alpha 1 anti trypsin is the most abundant protease inhibitor and is present in plasma at concentrations of 2-4mg/ml (Heimburger 1974). It is produced principally in the liver but also by macrophages, monocytes and recently production in PMNs has been demonstrated (du Bois et al 1991).

It has a broad range of activity against plasma proteins. Along with alpha 2 macroglobulin it accounts for approximately 90% of the plasma total inhibitory capacity, but is most active in the inhibition of serine proteinases (Ohlsson 1978). Alpha 1 antitrypsin has a molecular weight of 55,000. It has a 1:1 molar combining ratio with elastase which appears to have a higher affinity for alpha 1 antitrypsin than alpha 2 macroglobulin; 92% binding of elastase as opposed to 8% with alpha 2 macroglobulin. The complex between alpha 1 antitrypsin and elastase possesses no residual protease activity since alpha 1 antitrypsin binds the active site of elastase.

The half life of alpha 1 antitrypsin in the circulation is approximately 1 hour. Dissociation of the alpha 1 antitrypsin-elastase complex results in transfer of elastase to alpha 2 macroglobulin following which it is removed by cells of the reticulo-endothelial system. In the extra-cellular tissues the half life of these complexes is likely to be longer due to the much slower clearance.

1.9.2   Alpha 2 macroglobulin

Alpha 2 macroglobulin, the other principal protease inhibitor of serum, is present at concentrations of 1.5-4.2 mg/ml (Heimburger 1974). Like alpha 1 antitrypsin it is principally produced by the liver but its production in gingival macrophages has been demonstrated (Condacci et al 1988, Giannopoulou et al 1990). It has a molecular weight of 725,000 and is composed of 8 subunits of molecular weight 80,000 (Roberts et al 1974).

Alpha 2 macroglobulin combines irreversibly with elastase in a 2:1 molar ratio. There is no single binding site for elastase on alpha 2 macroglobulin, rather elastase combines with the central core of the molecule following its proteolytic attack of that region. This irreversible combination leads to a conformational change in the alpha 2 macroglobulin

which is subsequently treated as a foreign body and eliminated by monocytes. The alpha 2 macroglobulin/elastase complex retains limited proteolytic activity against low molecular weight proteins (Barrett and Starkey 1974, James 1980).

The interaction of protease with alpha 2 macroglobulin has certain characteristics (Barrett and Starkey 1974):

- Only active endopeptidases react

- The enzyme is irreversibly bound

- A molecule of alpha 2 macroglobulin can react with only one molecule of protease

- Bound enzyme is most inhibited with respect to large substrate molecules

- Bound enzyme is protected from other high molecular weight inhibitors

Alpha 2 macroglobulin has several important functions in the immune system. *In vitro* it binds not only to active proteases but also lymphokines. The possible role of alpha 2 macroglobulin in the immune system was reviewed by James (1980). Briefly, *in vitro* evidence suggests that it may be synthesised by lymphocytes, that it can alter the response of lymphoreticular cells to chemotactic and mitogenic agents thereby potentially affecting the recruitment and activation of macrophages and PMNs at inflammatory sites.

1.10   Aim of Study

The aim of the study was to examine the dynamics of degranulation of PMNs in the gingival crevice. It was postulated that an alteration in PMN degranulation dynamics may be useful as a marker of inflammatory periodontal disease rather than simple gingival inflammation. It was further postulated that the function and activity of elastase is locally altered by the presence of its inhibitors. This functional alteration may be indicated by a change in the relative levels of elastase and its inhibitors at different categories of sites.

GCF was collected from healthy, gingivitis and periodontitis sites within the same individual for the study. The PMN granule components elastase and lactoferrin, primary and secondary granule markers respectively, were identified in GCF. In addition the relationship of elastase to alpha 1 anti trypsin and alpha 2 macroglobulin, its principal inhibitors, at the same sites was measured. It was felt that by collecting GCF from different sites in the same individual some of the inter individual variability would be avoided.

CHAPTER 2

Clinical and Experimental Materials and Methods

Clinical Methods

2.1   Study Design

The study was cross sectional with 10 individuals examined and included for data and sample collection. Ethical committee approval was obtained for the study prior to its commencement. All individuals gave informed consent prior to their inclusion in the study. At the time of its inception the study was unique in the examination of PMN markers from different quality periodontal sites in the same individual.

2.2   Subject and site selection

Newly referred patients attending the Periodontal Department of Glasgow Dental Hospital and School were screened for inclusion in the study at their initial consultation visit.

All individuals were over 18 years of age and had clear medical histories with reference to conditions or treatments likely to alter the presentation or course of periodontal diseases. All were questioned regarding tobacco usage and were excluded if they admitted to its use. In addition to these systemic features, patients who had undergone periodontal treatments or used antibiotics in the previous 3 months were excluded. This was done in an attempt to avoid the effect of instrumentation and disruption on the periodontal environment when samples were collected.

All patients underwent a periodontal examination at which time 3 sites were identified for the collection of GCF. These sites required to be easily accessible and capable of isolation

from salivary contamination. The sites identified were classed according to gingival condition, probing depth and radiographic examination as:

Healthy   sites with no evidence of periodontal attachment loss, no bleeding on probing, no marginal gingivitis and periodontal pocketing of <3mm.

Gingivitis   no evidence of periodontal attachment loss, periodontal pocketing <3mm, bleeding on probing and marginal gingivitis.

Periodontitis   evidence of periodontal attachment loss as assessed clinically and radiographically, periodontal pocketing of >4mm, bleeding on probing and marginal gingivitis.

Following the identification of sites, arrangements were made to see the patients on a subsequent visit, prior to their first treatment session, for the collection of data and samples.

2.3   Clinical Measurements of Periodontal Status

An index is an objective method for measuring a clinical state. The use of an index facilitates the standardization of the degree of change or difference in the condition under observation. The choice of one index in preference to another depends on the aims and objectives of the situation and has been discussed extensively (Mandel 1974).

2.3.1   Plaque Indices

In the measurement of plaque and soft accumulations on teeth and restorations, indices estimate either the extent of tooth coverage or the thickness of deposits in relation to the gingival margin (Fischman 1986).

If the objective is to determine the efficacy of plaque removal or inhibition measures, then the measurement of changes in the area of tooth coverage may be desirable. If the objective in recording an index is to examine the relationship of plaque to the gingival condition, then it is more appropriate to employ an index system which weights the quantity of plaque at the gingival margin.

For the purposes of the present study the Plaque Index of Silness and Loe (Loe 1967) was used since it relates plaque deposits to the gingival tissues on the following scale:

0   no plaque present at the gingival area either visually or with a probe run along the tooth surface.

1   plaque present in a thin film at the gingival margin adherent to the tooth. This is not detected visually but by running a probe along the gingival area of the tooth surface.

2   Moderate accumulations of plaque and soft deposits in the gingival area, which can be seen with the naked eye.

3   Large accumulations of plaque and soft deposits in the gingival area, visible with the naked eye.

One criticism of this index is that it is not particularly sensitive to small variations in plaque levels. As the present study aimed principally to differentiate sites on the basis of gingival inflammation rather than plaque accumulation this was not considered to be of major importance.

2.3.2 Gingival Indices

As with indices of plaque, there are several techniques for observing the degree of gingivitis. As with plaque indices the selection of an appropriate index depends on the aims and objectives of the particular study.

The available indices rely on observations relating to gingival colour, gingival contour, bleeding on probing, the extent of gingival involvement or gingival crevicular fluid flow (Cianco 1986). They can be divided into those which report visual changes in the gingival tissue and those which rely not only on visual changes but also on the presence or absence of bleeding on probing in the gingival crevice or interdental region.

The recording of many indices causes disruption of the gingival crevice due to the insertion of a periodontal probe into it (reviewed by Ciancio 1986, Lobene et al 1986). This has 2 major effects; firstly if more than one examiner is involved in the collection of data trauma caused by the initial examiner can distort the site for subsequent examiners, secondly, the gingival crevice itself will be disrupted, most often by bleeding or the disturbance of subgingival plaque during recording. This has implications for the collection of GCF from that region with regard to the contamination of samples with blood or inaccurate microbiological samples.

The major clinical component of the present study involved the collection of samples of GCF. Care was taken in the selection of patients who had undergone no recent periodontal interventions and thus had undisturbed gingival crevices. The gingival index of Lobene et al (1986) is an index sensitive to small visual changes in gingival quality and it does not involve periodontal probing. This index had been used successfully in the department in previous studies (Adonogianaki 1992, Haerian et al 1995) where uncontaminated samples of GCF were required. The following scale is used:

0   no inflammation

1   mild inflammation; slight changes in colour, little changes in a portion of the
        gingival tissue but not involving the entire marginal or papillary unit.

2   mild inflammation as above but involving the entire gingival or papillary unit

3   moderate inflammation; glazing, redness, oedema and/or hypertrophy of the
        marginal gingival or papillary unit

4   severe inflammation; marked redness, oedema and/or hypertrophy of the marginal
        or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

2.3.3   Periodontal Probing

Periodontal probing is an established and widely used method for the assessment of periodontal status (Greenstein 1984, Lang et al 1990). It involves the insertion of a calibrated probe into the gingival sulcus or periodontal pocket, parallel to the long axis of the tooth root, to the point of resistance. A measurement of that depth either with reference to the gingival margin, the amelo-cemental junction or to a fixed point on the tooth or measuring stent is then made. The principal clinical limitation of periodontal probing is its ability to monitor tissue changes only retrospectively (Caton et al 1981, Listgarten 1980).

In cross sectional trials there is no requirement for reproducibility of probing depth at separated time points, despite this a reliable and reproducible probing technique is required. When clinical groupings are widely separated in terms of pocket depth the sensitivity of the technique to fractions of millimeters is not critical. In the present study the presence or absence of disease was based on a combination of clinical features. Site discrimination was on the basis of probing depth, attachment loss and the degree of superficial inflammation.

Probing depths were measured in millimetres using a calibrated Williams probe. The probe was inserted into the pocket in the long axis of the tooth until resistance was felt. The pocket depth was read visually and recorded.

2.3.4 Collection of GCF

Samples were collected using Whatmann grade 4 paper. Strips of 2mm by 13mm were cut and marked at a distance of 8mm from the end then autoclaved. This mark was an indication of the length of paper to be inserted between the beaks of the Periotron. All strips were handled using rubber gloves to avoid contamination by the operator and were stored in sterile glass bottles prior to use.

As previously discussed, Griffiths et al (1988) suggested that Whatmann grade 3 papers had the ideal properties for collection of GCF, however, previous experience in the department (Adonogianaki 1992) showed that zero readings could not be obtained on the Periotron with that paper.

The selected sites were isolated using a saliva ejector and cotton wool rolls to avoid contamination. Supragingival plaque was removed atraumatically using a curette. The site was dried using a gentle, coronally directed stream of air. Following this a paper strip was

inserted into the pocket orifice until light resistance was felt. The strip was left in situ for 30 seconds. Any strip visibly contaminated by blood was discarded and if the patient did not have an alternative, previously identified, site for sampling they were excluded from the trial. After removal from the sulcus, the strip was immediately placed between the beaks of the Periotron and the volume reading recorded. The strip was placed in a microcentrifuge tube, sealed to avoid evaporation and stored on ice prior to transfer to the lab for elution.

2.4 Sequence of collection

Samples and data were collected in the following sequence:

1. Plaque Index

2. Gingival Index

3. GCF collection

4. GCF volume determination

5. Probing pocket depth

All recordings and samples were obtained by one operator (M.C Murray) who had been previously calibrated in the recording of the indices and the collection of GCF as described.

Experimental Materials and Methods

2.5   Buffers

All chemicals, unless otherwise stated, used for the preparation of buffers were supplied by BDH Laboratory Supplies, Poole, Dorset.

2.5.1   Phosphate Buffered Saline

PBS used throughout the experiments had the following composition:

8g	NaCl
1.44g	$Na_2HPO_4.2H_2O$
0.2g	KH_2PO_4
0.2g	KCl

made up to 1 litre with distilled H_2O

2.5.2   Coating Buffer

Coating buffer was used for the carriage of reagents and samples in the coating phases of the experiments and in the pre-coating wash stage:

1.5g	Na_2CO_3
2.93g	$NaHCO_3$

dissolved in 800ml of distilled water.

This solution was made up to almost 1 litre with distilled water and the pH corrected to 9.6. Distilled water was then added to give a final volume of 1 litre. Coating buffer was stored at 4°C for a maximum of 1 week prior to use.

2.5.3   Incubation Buffer

The following incubation buffer was used as the carrier for all experiments during the incubation phase:

8g	NaCl
1.44g	$Na_2HPO_4.2H_2O$
0.2g	KH_2PO_4
0.2g	KCl
0.5g	Tween 20 (Sigma Chemical Company, Poole, Dorset)

This was made up to 1 litre in distilled H2O.

1g of BSA (Sigma, St Louis, USA) was layered over the surface and allowed to dissolve.

The buffer was stored at 4°C for a maximum of 1 week prior to use.

2.5.4   Wash Buffer

Wash buffer was used between the different stages of incubation to remove the unbound antibody or antigen from the wells:

80g	NaCl
14.4g	$Na_2HPO_4.2H_2O$

2g	KH_2PO_4
2g	Kcl
5g	Tween 20 (Sigma Chemical Company, Poole, Dorset)

Dissolved in 1 litre of distilled water. This was stored at room temperature and diluted 1 in 10 immediately prior to use with distilled water.

2.5.5 Substrate Buffer

For the final visualisation of the reaction in the wells, substrate buffer was used:

0.35g	Na_2HPO_4
0.2375g	Citric acid

this was dissolved in 25ml of distilled water and subsequently

10mg	(1 tablet) O-phenylenediamide dihydrochloride(OPD) (Sigma Immuno Chemicals, St Louis, USA)
10ul	H_2O_2

were added. This substrate was prepared in a fume cupboard immediately prior to use as OPD is toxic and the buffer unstable.

2.5.6 H_2SO_4

H_2SO_4 was used to stop the reaction of the substrate buffer on all occasions. This was prepared in the fume cupboard to give a solution with a final concentration of 1M

2.6   Periotron

2.6.1   Calibration of the Periotron

The Periotron 6000 GCF was used (Harco Electronics, Winnipeg, Canada). Calibration of the Periotron was undertaken as described by Adonogianaki (1992). Briefly, the Periotron was calibrated at every clinical session with PBS to take into account the effect of changes in atmospheric humidity. Known volumes of PBS were delivered onto Whatmann grade 4 paper strips, identical to those used for sample collection, using a Hamilton syringe. Volumes between 0.1 and 0.9ml were used in duplicate. Mean values of these recordings were used in a linear regression analysis from which the slope and intercept were used to determine GCF volumes using a Quattro software package.

2.7   Elution of samples

Samples were eluted into 1 ml of incubation buffer within 1 hour of collection. This was carried out over 1 hour at room temperature on a rotating mixer. Following this initial elution the sample was divided into 200ul aliquots and stored at -30°C in microcentrifuge tubes until use in the ELISA.

A standard elution technique was used. It was not possible to divide the elutions between coating and incubation buffers. This was not ideal with regard to the elastase assay where the samples were being used at the initial coating stage.

Sample dilution prior to capture on the plate was carried out to ensure values which fell within the working range of the assay as described. This sample dilution allowed for the

quantification of all substances from aliquots of the same sample. The dilutions were carried out as indicated on table 2.1.

2.8   Quantification of lactoferrin, alpha 1 anti trypsin, alpha 2 macroglobulin and elastase

2.8.1   Plates

All of the ELISA experiments were carried out using 96 well, Immulon 4 microtiter plates (Dynatech Laboratories, Virginia, USA). These plastic plates are formulated to give high protein absorption and binding, with low well-to-well variation.

These plates had been used and validated previously within the department for the ELISA tests being carried out.

2.8.2   Lactoferrin, alpha 1 antitrypsin and alpha 2 macroglobulin ELISAs

These were as described by Adonogianaki (1992) and are outlined on table 2.2

All were sandwich ELISAs using primary and secondary antibodies for the capture and identification respectively of the agent under investigation in GCF sample.

Table 2.1   **GCF sample dilutions after elution from strips and prior to use in the ELISA**

GCF Volume (v) in uL	Assay		
	alpha 2 macroglobulin	alpha 1 antitrypsin and lactoferrin	elastase
	Dilution factor		
v <- 0.1	1 / 2.5	1 / 5	undiluted
0.1 < v <- 0.2	1 / 5	1 / 10	undiluted
0.2 < v <- 0.4	1 / 10	1 / 20	undiluted
0.4 < v <- 0.8	1 / 20	1 / 40	undiluted
0.8 < v	1 / 40	1 / 80	undiluted

Table 2.2   Steps and reagents for lactoferrin, alpha 2 macroglobulin and alpha 1 antitrypsin ELISAs

STEPS	REAGENTS
1. Wash with 200uL/well three times, CB and dry	
2. Coat with 100uL/well specific antiserum. Incubate overnight at 4°C	Goat antisera to: lactoferrin and alpha 2 macroglobulin 1:6000 in CB; alpha 1 antitrypsin 1:3000 in CB
3. Wash with 200uL/well WB 6 times/well, 3 times and dry	
4. Add 100uL/well standard antigen or eluted sample. Incubate for 2 hours 37°C	Standard lactoferrin, alpha 2 macroglobulin or alpha 1 antitrypsin in IB
5. Wash with 200uL/well WB 6 times/well, 3 times and dry	
6. Incubate with 100uL/well second specific antiserum overnight, 4°C	Rabbit antisera to: lactoferrin, alpha 2 macroglobulin or alpha 1 antitrypsin 1:4000 in IB
7. Wash 200uL/well WB 6 times/well, 3 times and dry	
8. Add 100uL/well HRP conjugated IgG. Incubate at 37°C for 90 mins	HRP anti rabbit IgG(goat), 1:4000 in IB
9. Wash with 200uL/well WB 6 times/well, 3 times and dry	
10. Add 100uL/well substrate buffer and incubate at room temp (alpha 1 antitrypsin and alpha 2 macroglobulin ˷15 mins, lactoferrin ˷20 mins.	
11. Stop colour development with the addition of H_2SO_4 1M, 50uL/well. Read at 490nm.	

Alpha 2 macroglobulin, Goat anti alpha macroglobulin, rabbit anti alpha 2 macroglobulin, alpha 1 antitrypsin, goat anti alpha 1 antitrypsin and rabbit anti alpha 1 anti trypsin were all supplied by Sigma Immuno Chemicals (St Louis, USA). Lactoferrin was supplied by Calbiochem. Goat anti lactoferrin and rabbit anti lactoferrin were supplied by Nordic. HRP conjugated anti rabbit IgG was supplied by ICN. OPD was supplied by Sigma (St Louis, USA).

Sample dilution prior to capture on the plate was carried out to ensure values which fell within the working range of the assay as described. The working ranges of the assays are displayed in Table 2.3.

The recovery rate of the 3 agents had been previously determined and was as shown in Table 2.4.

All plates had peripheral control wells and incorporated serial dilutions of pure agent in duplicate. The mean of the readings for these dilutions was used in a regression analysis the slope and intercept of which was used for the conversion of plate readings to the amount of agent in the sample. Wells at the edge of the plate were not used for sample quantification to avoid the edge effect (Tijssen 1988)

2.8.3 Elastase ELISA

An ELISA was devised for the detection and quantification of PMN elastase. Due to the difficulties in obtaining an anti PMN elastase antibody and the difficulty of working with a biotinylated polyclonal PMN elastase antibody, a direct ELISA was used, table 2.5. Purified PMN elastase was supplied by Sigma. Rabbit anti PMN elastase was supplied by Calbiochem, (La Jolla, CA). HRP conjugated anti rabbit IgG was supplied by ICN.

Table 2.3   **Working range of the standard antigen for the ELISA assays of elastase, lactoferrin, alpha 1 antitrypsin and alpha 2 macroglobulin.**

Assay	Working range ng/mL
Elastase	125 - 0.24
Lactoferrin	125 - 0.24
Alpha 1 antitrypsin	100 - 0.19
Alpha 2 macroglobulin	200 -0.39

Table 2.4   Recovery rates of alpha 2 macroglobulin, alpha 1 antitrypsin and lactoferrin in incubation buffer (IB). (From : Adonogianaki 1992)

	Concentration in 1mL of eluate ng/mL		Recovery rate
Analyte	strip (n=7)	IB (n=3)	
Alpha 2 macroglobulin	1520 +/- 52	1527 +/- 53	99.5%
Alpha 1 antitrypsin	1266 +/- 72	1273 +/- 41	99.4%
Lactoferrin	3763 +/- 232	4509 +/- 81	83.4%

As part of the study the recovery rate of elastase was determined. As samples had all been eluted into incubation buffer, comparisons were made between the recovery rate from incubation and coating buffers. Purified PMN elastase standard was used.

The following procedure was carried out; 1ul of stock purified PMN elastase at 1mg/mL was delivered into one of the following:

- on Whatman Grade 4 paper strips, 10 strips, subsequently eluted into 1ml of either incubation buffer (n=5) or coating buffer (n=5) at 22°C on a rotating mixer for 1 hour

- directly (n=10) either into 1ml of incubation buffer (n=5) or coating buffer (n=5) and rotated at 22°C on a rotating mixer for 1 hour

Delivering pure PMN elastase into both incubation and coating buffers enabled the determination of the reduction in coating which would be found using GCF samples in the assay. Following elution as described above, the samples were coated onto plates and the ELISA run as outlined in Table 2.5. The recovery rate of elastase from incubation buffer was greatly reduced, see Table 2.6, but was still well within the working range of the assay. Clinical samples used in the elastase ELISA were not diluted following initial elution prior to use in the assay due to this low recovery rate.

As with the ELISAs for lactoferrin, alpha 1 antitrypsin and alpha 2 macroglobulin, each plate incorporated peripheral control wells and serial dilutions of pure agent in duplicate. The mean of the duplicate measurements was used in a regression analysis the slope and intercept of which was used for the conversion of plate readings to the amount of agent in the sample.

Table 2.5   Steps and reagents for the elastase ELISA

STEPS

1. Wash with CB 200uL/well 6 times/well, three times and dry

2. Standard elastase in CB or eluted GCF sample added and incubated overnight at 4°C.

3. Wash with WB 200uL/well 6 times/well, three times and dry

4. Add Rabbit anti elastase at 1 in 1000 dilution in IB, incubate at 37°C for 2 hours

5. Wash with WB 200uL/well 6 times/well, three times and dry

6. Add HRP anti rabbit IgG at 1 in 10000 dilution in IB. Incubate at 37°C for 1 1/2 hours.

7. Wash with WB 200uL/well 6 times/well, three times and dry

8. Add OPD substrate 100uL/well, incubate for ~15mins at room temperature.

9. Reaction stopped by the addition of 50uL/well of H_2SO_4

The reaction was finally read at 490nm using a Dynatech MR 5000 plate reader

Table 2.6 :   Recovery rate of elastase from coating buffer and incubation buffer compared with elution directly into coating buffer

Method of delivery	Average concentration (ng/mL)	Percentage recovery rate
Direct to incubation buffer	1.2088	2.6%
Strip to coating buffer	0.8022	1.7%
Strip to incubation buffer	1.253	2.7%

2.9   Statistical analysis

Statistical analysis was carried out using a Minitab release 9.2 package.

For all analysis the site was taken as the statistical unit. Data referring to alpha 1 antitrypsin, alpha 2 macroglobulin, lactoferrin and elastase were markedly skewed in their distribution. As a result, analysis was carried out on the log transformed data relating to all of the biochemical parameters with the exception of elastase. For elastase, even following transformation, the distribution was not normal and as a result non parametric tests were carried out in relation to this.

Since three comparisons were being made between the groups when using the 2 sample t test, the level of significance was taken as 0.025, thus compensating for the 3 comparisons (Brown and Swanson Beck, 1988)

CHAPTER 3

RESULTS

3.1   Clinical study

3.1.1   Sample Description

A total of 10 subjects were recruited to the study, 3 males and 7 females, age range 37-51 years. Each subject had GCF samples collected in a standardised way, as described in Chapter 2, from all 3 categories of sites i.e. healthy, gingivitis and periodontitis.

3.1.2   Plaque Index

The mean plaque index at healthy sites was 0.1 (range 0-1, SD 0.316), at gingivitis sites 0.9 (range 0-3, SD 1.101) and at periodontitis sites 1.7 (range 0-3, SD 0.823).

Using the 2 sample t test the healthy group showed a statistically significant difference from the periodontitis group, $p<0.0001$. Differences between healthy and gingivitis sites, $p=0.052$, and gingivitis and periodontitis sites, $p=0.084$, were not significant.

3.1.3   Gingival Index

The mean gingival index at healthy sites was 0.1 (range 0-1, SD 0.316), at gingivitis sites 2.4 (range 1-3, SD 0.699) and at periodontitis sites 2.3 (range 1-3, SD 0.823)

Using the 2 sample t test significant differences were shown between healthy and gingivitis sites, $p<0.001$, between healthy and periodontitis sites, $p<0.001$ but not between gingivitis and periodontitis sites, $p=0.77$.

This finding confirmed the clinically observed separation between healthy and inflamed sites in either gingivitis or periodontitis categories on the basis of the modified gingival index. In addition, the level of superficial inflammation as judged clinically, was similar in gingivitis and periodontitis sites.

3.1.4 Pocket Depth

The mean pocket depth at healthy sites was 0.9mm (range 0-2, SD 0.699), at gingivitis sites 2.6mm (range 2-3mm, SD 0.516) and at periodontitis sites 5.8mm (range 4-10mm, SD 1.1814).

Using the 2 sample t test, significant differences were found between all groups; healthy compared with gingivitis $p<0.001$, healthy compared with periodontitis $p<0.001$ and gingivitis compared with periodontitis $p=0.003$.

The clinical groups showed almost complete separation, again confirming the clinical differences between the groups.

3.1.5 Gingival Crevicular Fluid Volume

The mean GCF collected over 30 seconds at healthy sites was 0.1014ul (range 0.005-0.618, SD 0.1821), at gingivitis sites 0.1474ul (range 0.014-0.331, SD 0.0999) and at periodontitis sites 0.4213 (range 0.005-0.7530, SD 0.2204).

Data for GCF volume were markedly skewed. Log transformation produced a normal distribution and thus satisfied the distributional requirements of the 2 sample t test. Despite the tendency for GCF volume to increase from healthy to gingivitis sites this was not significant, $p=0.13$. Between healthy and periodontitis sites a significant difference was present, $p=0.0099$, but was absent when gingivitis and periodontitis sites were compared, $p=0.11$.

These data confirmed the tendency for GCF volume to be increased at sites with inflammation and increased pocket depth.

The above clinical results, particularly relating to pocket depth and gingival inflammation, confirm that the separation and selection on clinical grounds at entry into the study was valid. In addition to the measurement of superficial gingival inflammation, using the modified gingival index and pocket depth, all sites in both the gingivitis and periodontitis groups exhibited bleeding on probing into the gingival crevice. Inclusion of this feature reduces the possibility that a non active site is chosen for sampling (Lang et al 1990).

3.2   PMN Granule Markers

3.2.1 Elastase (Primary PMN granule)

As the distribution of values relating to elastase could not be normalised following log transformation, analysis was carried out using the non parametric Mann Whitney test.

Absolute Amounts (ng/30 second sample)

The mean amount of elastase detected at healthy sites was 0.475ng (range 0-1.1216ng, SD 0.427), at gingivitis 1.56ng (range 0-11.6ng, SD 3.35) and at periodontitis sites 0.363ng (range 0.1198-0.8462, SD 0.2558).

Although there was a slight tendency for elastase levels to be higher at gingivitis sites compared with healthy, p=0.497, and at periodontitis sites, p=0.1859 compared with healthy, this was not significant. In addition gingivitis and periodontitis sites could not be discriminated on the basis of the amount of elastase, p=0.7333.

Concentration (ng/ul/30 second sample)

The mean concentration of elastase at healthy sites was 12.59ng (range 0-57.1, SD 17.2), at gingivitis sites 9.57ng (range 0-53.7, SD 16.04) and at periodontitis sites 3.35ng (range 0.26-23.95, SD 7.34).

Again, no significant differences were found between healthy and gingivitis sites, p=0.7055 or healthy and periodontitis sites, p=0.3447. The difference between gingivitis and periodontitis sites just failed to reach significance, p=0.0452. Gingivitis sites had higher mean concentration than periodontitis.

For all the sampled groups the range of values for both absolute amounts and concentration of elastase was very wide with large standard deviations being present. This may explain why no statistically significant differences could be detected between the different types of site. Larger numbers of subjects would be required to confirm whether the absence
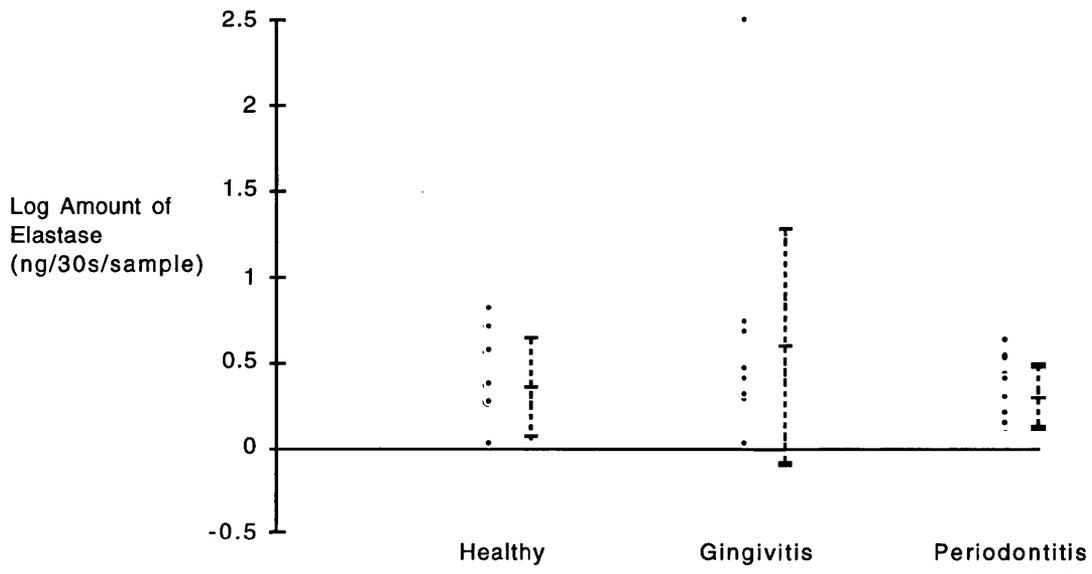
Figure 3.1:   Absolute amount of elastase/30 second sample of GCF

of any difference was due to the small sample size or was in fact a biological feature.

3.2.2 Lactoferrin (Secondary PMN granule)

Absolute amounts (ng/30 second sample)

The mean amount of lactoferrin detected at healthy sites was 106ng (range 2.3-649, SD 193.9), at gingivitis sites 87 (range 22.7-288, SD 77.8) and at periodontitis sites 620 (range 3-1518, SD 538).

Statistically significant differences were present using the 2 sample t test, between healthy and periodontitis sites, $p=0.0021$, but not between healthy and gingivitis, $p=0.31$, or gingivitis and periodontitis sites, $p=0.052$.

Concentration (ng/ul/30 second sample)

The mean concentration of lactoferrin detected at healthy sites was 2288ng (range 47-14782, SD 4446), at gingivitis sites 804 (range 208-2217, SD 626) and at periodontitis sites 2368 (range 7-11633, SD 3354).

No significant differences were present between any of the groups in relation to lactoferrin concentrations using the 2 sample t test; healthy compared with gingivitis $p=0.79$, healthy compared with periodontitis $p=0.71$ and gingivitis compared with periodontitis $p=0.49$.
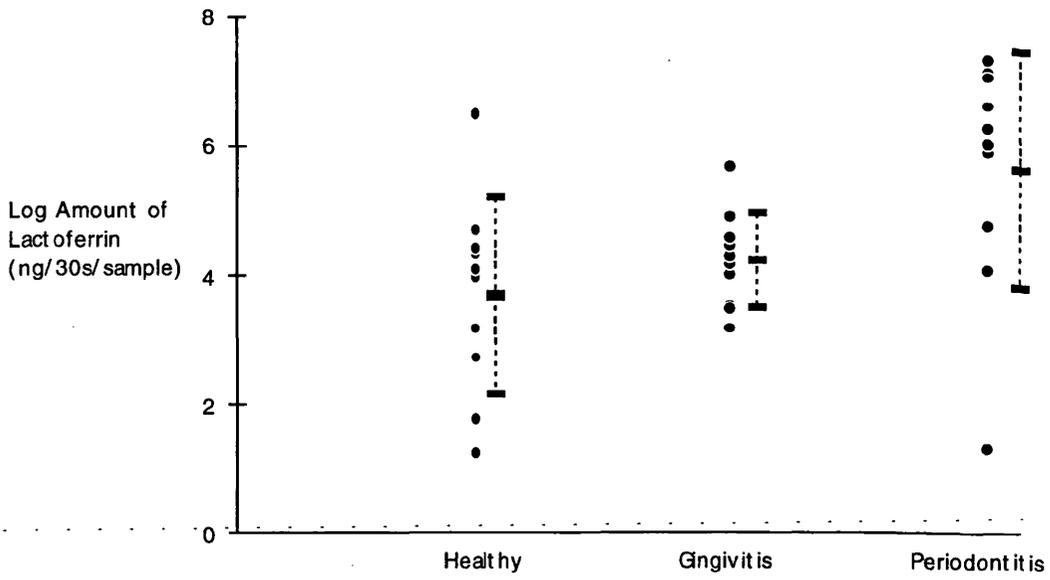
Figure 3.2:   Absolute amount of lactoferrin/30 second GCF sample

These data confirm previous findings in our laboratory that the amount of lactoferrin recovered in GCF is related to the GCF volume and that the concentration remains constant at different disease levels.

3.2.3 Relationship of Elastase to Lactoferrin as an indicator of Primary and Secondary PMN granule release

The relationship of primary and secondary granule products at all categories of disease sites was examined. This data was not normally distributed and could not be normalised following log transformation, thus the non-parametric Mann Whitney test was used in the analysis of the raw data.

The mean lactoferrin to elastase ratio at healthy sites was 133.3 (range 2.3-533, SD 165), at gingivitis sites 136.8 (range 7.7-272.1, SD 102.7) and at periodontitis sites 1973 (range 13-4140, SD 1485).

No significant differences were found between healthy and gingivitis sites, p=0.4274. However between gingivitis and periodontitis, p=0.0073 and between healthy and periodontitis sites, p=0.0058, highly significant differences were present with a 10 fold increase in the amounts of lactoferrin relative to elastase present in the samples.

This difference in the relationship of elastase to lactoferrin may be an indicator of altered PMN granule release dynamics at periodontitis sites and may be a useful discriminator between gingivitis and periodontitis.

3.3   Protease Inhibitors

3.3.1 Alpha 1 anti trypsin

Absolute amounts (ng/30 second sample)

At healthy sites the mean amount of alpha 1 anti trypsin detected was 112.5ng (range 1.7-369.3, SD 138.3), at gingivitis sites 485ng (range 146-1410, SD 403) and at periodontitis sites 707 (range 97-1482, SD 448).

An increase in the amount of alpha 1 anti trypsin detected was seen from healthy through gingivitis to periodontitis sites. The 2 sample t test showed significant differences between healthy and gingivitis sites, p=0.0033, and between healthy and periodontitis sites, p=0.0009 but not between gingivitis and periodontitis sites, p=0.26

Concentrations (ng/ul/30 second sample)

The mean concentration of alpha 1 anti trypsin at healthy sites was 2615 (range 35-10176, SD 3616), at gingivitis sites 4993 (range 941-20774, SD 5766) and at periodontitis sites 3469 (range 678-19362, SD 5639).

Using the 2 sample t test no significant differences were found between healthy and gingivitis sites, p=0.053, healthy and periodontitis sites, p=0.25 and gingivitis and periodontitis sites, p=0.19. Again however, there was a tendency for the concentration of
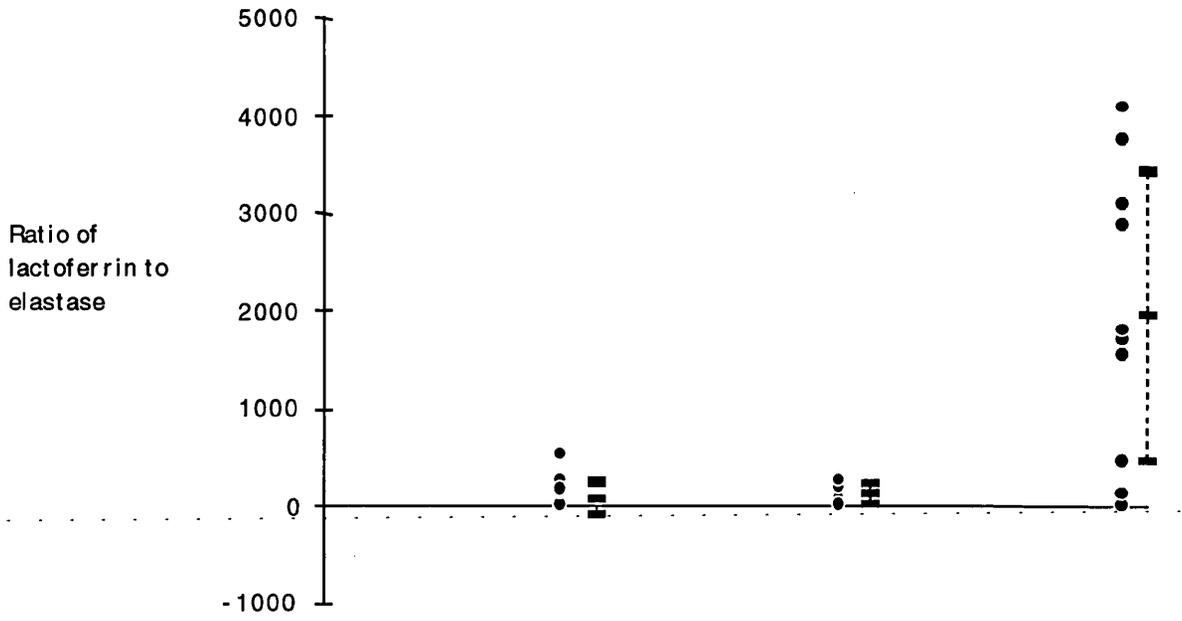
Figure 3.3:   Ratio of primary and secondary granule markers in GCF
samples

alpha 1 anti trypsin to be greater at gingivitis sites than at either healthy or periodontitis sites.

3.3.2 Alpha 2 macroglobulin

Absolute amounts (ng/30 second sample)

The mean amount of alpha 2 macroglobulin detected at healthy sites was 46.4ng (range 3.3-181.5, SD 56.3), at gingivitis sites 594 (range 49-1958, SD 595) and at periodontitis sites 636 (range 31-3508, SD 1041) A sharp increase in the amount of inhibitor present at gingivitis and periodontitis sites was seen.

The 2 sample t test showed significant differences between healthy and gingivitis sites, p=0.0001 and between healthy and periodontitis sites, p=0.0008 but not between gingivitis and periodontitis sites, p=0.66.

Concentration (ng/ul/30 second sample)

The mean concentration of alpha 2 macroglobulin at healthy sites was 1076 (range 66-4935, SD 1531), at gingivitis sites 10961 (range 279-75783, SD 23071) and at periodontitis sites 2163 (range 110-9040, SD 3010).

Increased concentrations of inhibitor were present at gingivitis sites.
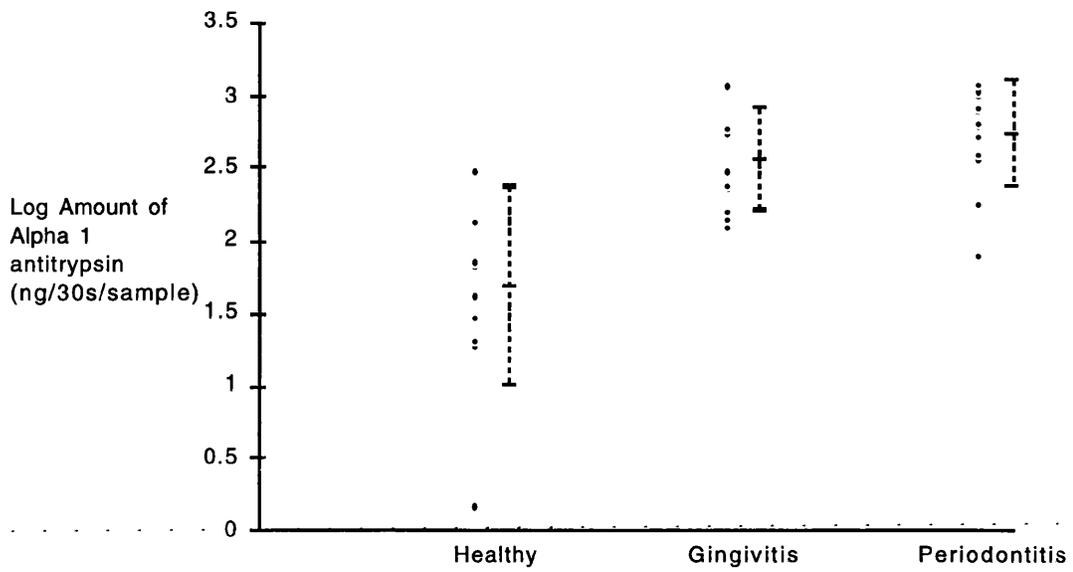
Figure 3.4: Absolute amount of alpha 1 antitrypsin/30 second GCF
sample

The 2 sample t test showed significant differences between healthy and gingivitis sites, p=0.0084 and a tendency to a difference between gingivitis and periodontitis sites, p=0.076 but no difference between healthy and periodontitis sites, p=0.25.

3.3.3 Relationship of alpha 1 anti trypsin and alpha 2 macroglobulin

The relationship of alpha 1 anti trypsin to alpha 2 macroglobulin was calculated for each site to determine if any change occurred in the relative amounts of these inhibitors at different types of sites.

Again, the data were skewed but had a normal distribution following log transformation. Thus, log transformed data were used in parametric analysis.

At healthy sites the mean alpha 1 anti trypsin/alpha 2 macroglobulin ratio was 2.61 (range 0.526-5.758, SD 1.676), at gingivitis sites 1.264 (range 0.274-3.41, SD 0.909) and at periodontitis sites 3.16 (range 0.37-12.65, SD 3.63).

Although there was a tendency for the amount of alpha 1 anti trypsin relative to alpha 2 macroglobulin to be higher at healthy sites, p=0.055 and periodontitis sites, p=0.090, than at gingivitis sites; no difference was detected between healthy and periodontitis sites, p=0.98.
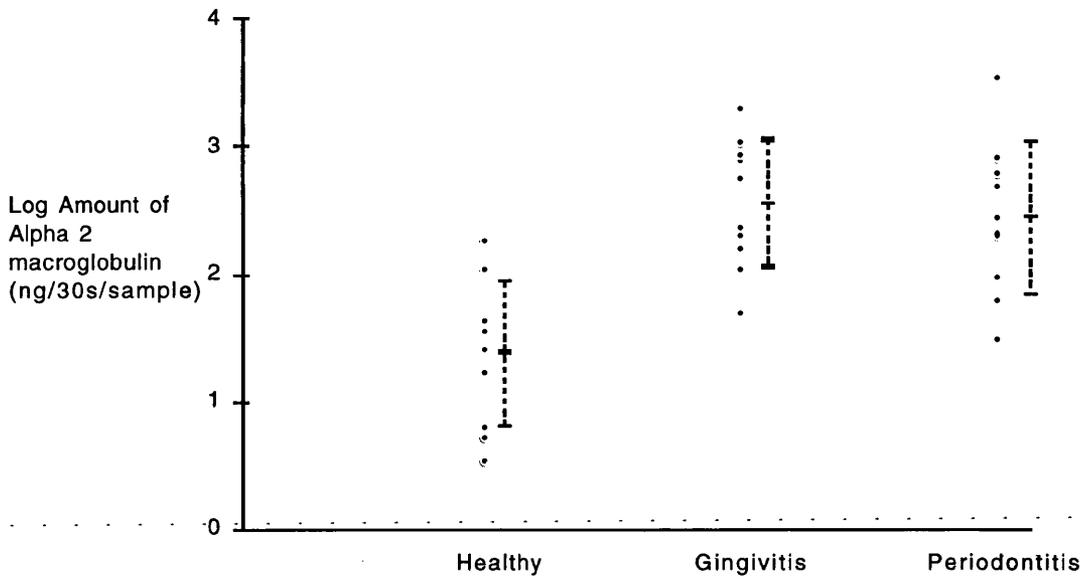
Figure 3.5:   Absolute amount of alpha 2 macroglobulin/30 second GCF
sample
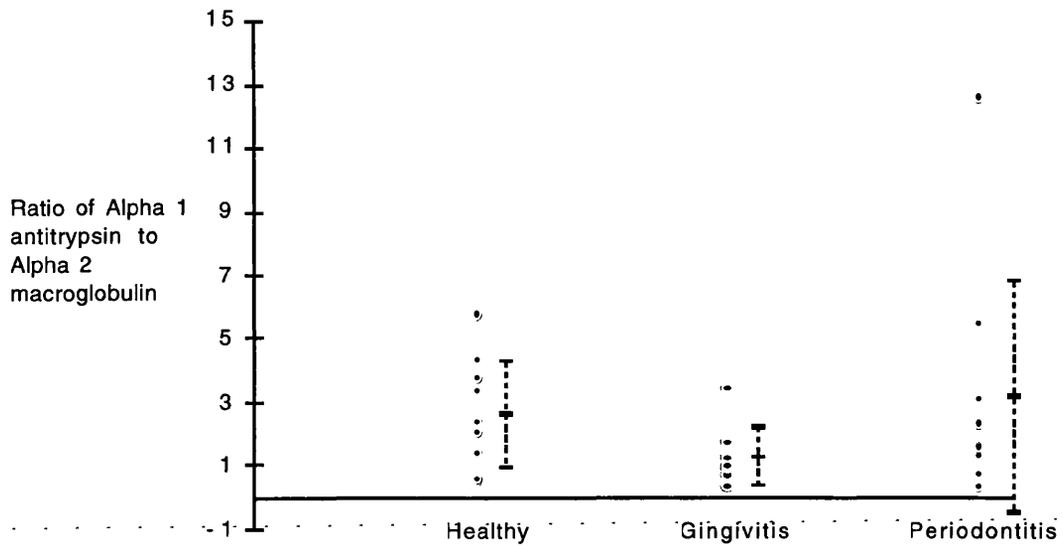
Figure 3.6: Relationship of protease inhibitors at different categories of sites in GCF samples

3.4 Relationship of elastase to its inhibitors

In an attempt to display potential differences between the relative levels of inhibitors and elastase at different disease sites, the ratio between elastase and its inhibitors was calculated for each site.

As the data were skewed, and following log transformation did not assume a normal distribution, the non parametric Mann Whitney test was used in the analysis.

3.4.1 Alpha 1 anti trypsin/Elastase

At healthy sites the mean alpha 1 anti trypsin/elastase ratio was 179.2 (range 1.7-874, SD 261.3), at gingivitis sites 728 (range 33-1583, SD 565) and at periodontitis sites 2464 (range 574-6578, SD 565).

There was a marked increase in the amount of inhibitor present relative to elastase from healthy through to periodontitis sites. Significant differences were detected between healthy and gingivits sites, p=0.0091, healthy and periodontitis sites, p=0.0003 and gingivitis and periodontitis sites, p=0.0028.

Alpha 1 anti trypsin binds elastase and completely inhibits its proteolytic ability. The finding that there is a relative increase in the amount of alpha 1 anti trypsin at sites of inflammation and attachment loss tends to support an attempt by the host to protect the tissues from the effects of elastase and potential tissue degradation.

3.4.2 Alpha 2 macroglobulin/Elastase

The mean ratio of alpha 2 macroglobulin/elastase at healthy sites was 72.4 (range 3.3-263, SD 82.5), at gingivitis sites 858 (range 14-2090, SD 708) and at periodontitis sites 2829 (range 90-17547, SD 5301).

Again, there was a large increase in the amount of inhibitor relative to elastase from healthy through to periodontitis sites. Significant differences were present between healthy and gingivitis sites, p=0.0028, and healthy and periodontitis sites, p=0.0008, but not between gingivitis and periodontitis sites, p=0.4274.

Alpha 2 macroglobulin binds elastase and inhibits some, but not all, of its protease activity. Again, a relative increase in the amount of the inhibitor at sites of inflammation would support an attempt by the host to protect the tissues. However, unlike alpha 1 anti trypsin where the relative amount increases from gingivitis to periodontitis, this is not seen for alpha 2 macroglobulin. This is confirmed below when the correlation between alpha 2 macroglobulin and elastase is seen to be lower than between alpha 1 anti trypsin and elastase.

3.5   Correlations between clinical and biochemical parameters

The correlations between the various clinical and biochemical parameters used in the study were examined. Only that data which was continuous was used in the correlation. Because of the skewed distribution of the data, values relating to alpha 1 anti trypsin, alpha 2 macroglobulin, elastase, lactoferrin and GCF volume were all log transformed. Where pocket depth was included in the correlation it was non transformed. The correlation analysis was carried out on stacked data.

Correlations were found between :

Alpha 1 anti trypsin with alpha 2 macroglobulin r=0.861

Alpha 1 anti trypsin with elastase, r=0.582

Alpha 1 anti trypsin with lactoferrin, r=0.572

Alpha 1 anti trypsin with GCF volume, r=0.636

Alpha 1 anti trypsin with pocket depth, r=0.610

Alpha 2 macroglobulin with GCF volume, r=0.454

Alpha 2 macroglobulin with pocket depth, r=0.430

Lactoferrin with GCF volume, r=0.697

Lactoferrin with pocket depth, r=0.626

GCF volume with pocket depth, r=0.708

Other correlations which were present were of r less than 0.4 and as a result were not reported.

If the desired level of correlation is set as that established between GCF volume and pocket depth, then the correlation between alpha 1 anti trypsin and alpha 2 macroglobulin is extremely high. It is considered that these inhibitors, although principally produced in the liver and derived in GCF from serum, are locally produced at sites of inflammation. The correlation would suggest that the local production of the two inhibitors is not completely, if at all, mutually independent.

Another interesting finding is of the poorer correlation between elastase and alpha 2 macroglobulin, r=0.377, than between elastase and alpha 1 anti trypsin, r=0.582. Given the fact that in our samples the relative amounts of alpha 1 anti trypsin and elastase, but

not alpha 2 macroglobulin and elastase at a site could distinguish not only between healthy and gingivitis sites or healthy and periodontitis sites but also between periodontitis and gingivitis sites, this combination of elastase and alpha 1 anti trypsin may be the one which is of more importance in disease differentiation than that of elastase and alpha 2 macroglobulin.

Table 3.1 : **Clinical Characteristics of sampled sites** (mean and standard deviation)

Parameter	Healthy	Gingivitis	Periodontitis
Gingival crevicular fluid volume (uL)	0.1014 sd 0.1821	0.1474 sd 0.0999	0.4213 sd 0.2204
Plaque Index	0.100 sd 0.316	0.900 sd 1.101	1.700 sd 0.823
Gingival Index	0.100 sd 0.316	2.400 sd 0.699	2.300 sd 0.823
Probing Depth(mm)	0.900 sd 0.699	2.600 sd 0.516	5.800 sd 1.814

Table 3.2: **PMN granule components** (mean and standard deviation)

Parameter	Healthy	Gingivitis	Periodontitis
Elastase (ng/30s)	0.3500 sd 0.2903	0.598 sd 0.695	0.2947 sd 0.1797
Elastase (ng/uL/30s)	64.0 sd 178.3	10.05 sd 15.85	3.35 sd 7.33
Lactoferrin (ng/30s)	3.671 sd 1.528	4.213 sd 0.738	5.654 sd 1.847
Lactoferrin (ng/uL/30s)	2333 sd 4447	778 sd 649	2368 sd 3353
Lactoferrin/ Elastase ratio	133 sd 165	136 sd 102.7	1973 sd 1485

Table 3.3 : Protease Inhibitors (mean and standard deviation)

Parameter	Healthy	Gingivitis	Periodontitis
Alpha 1 antitrypsin (ng/30s)	112.5 sd 138.3	485 sd 403	707 sd 448
Alpha 1 antitrypsin (ng/uL/30s)	2615 sd 3616	4993 sd 5766	3469 sd 5639
Alpha 2 macroglobulin (ng/30s)	46.4 sd 56.3	594 sd 595	636 sd 1041
Alpha 2 macroglobulin (ng/uL/30s)	1076 sd 1531	10961 sd 23071	2163 sd 3010
Alpha 2 macroglobulin/ elastase ratio	72.4 sd 82.5	858 sd 708	2829 sd 5301
Alpha 1 anti trypsin/ elastase ratio	179.2 sd 261.3	728 sd 565	2464 sd 565
Alpha 1 anti trypsin/ alpha 2 macroglobulin ratio	2.61 sd 1.676	1.264 sd 0.909	3.16 sd 3.63

Table 3.4   Correlations of markers

	Alpha 1 anti trypsin	Alpha 2 macroglobulin	Lactoferrin	GCF volume
Alpha 1 antitrypsin	<0.4	0.861	0.572	0.636
Alpha 2 macroglobulin	0.861	<0.4	<0.4	0.454
Elastase	0.582	0.377	<0.4	<0.4
Pocket Depth	0.610	0.430	0.626	0.708
Lactoferrin	0.572	<0.4	<0.4	0.697
GCF volume	0.636	0.454	0.697	<0.4

CHAPTER 4

DISCUSSION

4.1　Study Design Considerations

The principal aim of the study was to investigate potential differences in PMN granule components and levels of protease inhibitors in GCF at sites of periodontitis compared with health and gingivitis, with a view to highlighting site related differences. With that in mind a cross sectional study design was chosen.

Cross sectional studies collect information at one time point, at several different sites which are related. In this study the clinical characteristics determined the classification of the site on entry. The aim of site and candidate selection was to select periodontally active and undisturbed sites to compare with shallow inflamed and non inflamed sites. Sites which were designated as periodontitis sites on the basis of bone loss and pocket depth had marginal gingivitis and exhibited bleeding on probing to the base of the crevice. These clinical indicators were used as the best indicators of ongoing disease activity (Lang et al 1986). Candidates for sampling had received no periodontal interventions in the preceding 3 months. This reduced the potential for conditions in the gingival crevice to be influenced by instrumentation which affects GCF composition (Skapski and Lehner 1976). Finally, by collecting related groups of samples from sites within individuals some of the compounding effects of systemic related variants, such as tobacco smoking, diabetes and drug therapy were reduced.

In the study of PMN function in periodontal diseases peripheral blood is often used due to the difficulty in collection and retrieval of crevicular PMNs. As discussed by Wilton (1989) these peripheral blood PMNs may not be representative of those in the crevice, especially in view of the site specific nature of disease which is clinically observed (Socransky et al 1987). As a result it may be inappropriate to extrapolate from *in vitro* findings with peripheral blood PMNs, as has been done (Gustafsson et al 1994), to the *in*

vivo situation of the gingival crevice. Thus, in the study of PMN dynamics, locally collected PMN markers were assayed.

As the study involved the examination by ELISA of 4 GCF components, including protease inhibitors, a GCF paper strip sampling technique, rather than a gingival washing technique was used to collect samples as it is reliable and relatively easy to perform. Every effort was made during sample collection to obtain uncontaminated strips for the laboratory studies. This was achieved by isolating teeth during sampling and by discarding strips visibly contaminated with blood.

4.2   Choice of Laboratory Methods

Three of the assays had been developed and validated previously in our laboratory, the assays for lactoferrin, alpha 1 anti trypsin and alpha 2 macroglobulin (Adonogianaki 1992). As will be discussed later, the final assay for elastase was developed for the present study.

Most studies relating to GCF elastase measure elastase-like activity rather than the antigenic elastase levels. As the study was attempting to compare primary and secondary PMN granule components it was appropriate that the same method of assaying be used for both. Such measurement is complicated by the binding of elastase to protease inhibitors in GCF, which means that the level of elastase recorded may not directly reflect that released by PMNs. However, the measurement of elastase-like activity in GCF is also complicated by problems of potential cross reaction of other proteases with the experimental substrate. What would be ideal would be the combined measurement of antigenic elastase and elastase- like activity in the same sample (Cox 1995). Combination assays of that nature however encounter problems relating to the suitability of elution

techniques for each of the different assays used, which to some extent was the case with the assay developed for elastase. This approach may necessitate the collection of more than one GCF sample at each site, the composition of these samples would not be similar (Skapski and Lehner 1976).

The ELISA technique was used to assay for all GCF markers. ELISA is a technique by which antibody combined with antigen is detected following conjugation with a colourometric substrate. The colour change is monitored visually and correlates with the amount of antigen present in the original sample. Non competitive assays involve the use of antigen or antibody coated onto the solid phase. The final antibody is labelled to react with the substance used for visualisation in the system. The solid phase is generally a plastic, either polystyrene or PVC, available as plates or beads.

The level of marker recorded by ELISA is a measure of the amount of antigenic material detected in the sample. With regard to PMN granule components this is potentially from several sources. It may be from granule contents released in the gingival connective tissues and diffusing into the gingival crevice; from granule contents released into the gingival crevice prior to sampling; and from granules which are disrupted either during GCF collection or during the preparation and storage of samples. It was assumed that the amount of disruption caused to the PMN during sample elution involving the use of buffer containing TWEEN, which is a detergent, would affect primary and secondary granules in a similar way. This may not be the case. However any differential release of granular components during collection, elution and storage is not easily quantifiable using standard GCF collection methods. This is because GCF contains not only tissue and cell products but intact and functional cells which can be disrupted at any stage during handling.

Initially a sandwich ELISA for elastase was attempted. These assays have the advantage of being more sensitive and specific. In addition, following collection of samples GCF was eluted into incubation buffer prior to storage rather than into coating buffer. During its development several problems were encountered. The sources of PMN elastase antibodies were extremely limited and we were unable to locate two separate antibodies that could be used for ELISA. As a result we prepared a biotinylated anti PMN elastase and developed a sandwich ELISA utilising this as a second antibody. This biotinylated antibody was extremely unstable. By the time the working range for each new batch of antibody had been determined the antibody was no longer active. Plate readings for clinical samples using the biotinylated antibody were either extremely low or not detectable.

To overcome these problems an assay was developed using GCF samples as coating agents. The reduction in detectability was determined by comparing readings of purified antibody in coating buffer and incubation buffer eluted from paper strips. These eluted samples were then coated onto plates and used in the assay under the same conditions. This showed an average reduction in recovery of elastase to around 2.7%. In a recent paper Gustafsson (1996) reported that he had failed to recover uncomplexed PMN elastase from Periopapers. The present study recovered some PMN elastase, but like the study of Gustafsson (1996) this was much less than for other proteins examined. This greatly reduced sensitivity in the detection of elastase is a weakness, however the levels of elastase recorded were still within the working range of the assay. In addition, the elution pattern from paper strips of a commercially pure PMN elastase may not accurately reflect that of PMN elastase in GCF. In the reporting of the results elastase was, as a result, examined with most interest as it related to other agents assayed.

The problems in the measurement of elastase by ELISA of its potential to bind to inhibitors in GCF could not be fully addressed by the sampling and assaying techniques used. As a result of the assay technique used, it is not possible to say what combination of elastase and inhibitor or pure elastase is being detected. If an alternative marker of

primary granules is not used, a future study should assess the detection rate of purified elastase and inhibitors in varying concentrations. This would enable further understanding of the dynamics, *in vitro,* of the binding of elastase to its inhibitors. Nevertheless, even when elastase inhibitor combinations are determined *in vitro*, elastase *in vivo* may be bound to other inhibitory molecules (Remold-O'Donnell et al 1989).

4.3   Primary and Secondary granule markers in GCF

We found a statistically significant increase in the absolute amount of lactoferrin in GCF between healthy and periodontitis sites but no difference in the concentration of lactoferrin at any category of site. The level of lactoferrin recorded at healthy and gingivitis sites was similar to those previously recorded (Adonogianaki et al 1993). The high correlations between lactoferrin, pocket depth and GCF volume is similar to that study. Lactoferrin in GCF has not been studied extensively but has been proposed as a marker of activated PMN migration from vessels into GCF (Adonogianaki et al 1993, Gustafsson et al 1994). Adonogianaki et al (1994) further examined the profile of lactoferrin in experimental gingivitis. They found a statistically significant increase in lactoferrin as inflammation developed and a rapid decrease once oral hygiene was resumed. They related this rapid drop to the removal of plaque and the resultant decrease in the chemotactic stimulus for PMN migration into the gingival crevice. For the purposes of this discussion lactoferrin is taken to reflect the number of PMNs present in the crevice at the time of sampling.

Elastase levels, measured by ELISA, in GCF reflect the amount of antigenic elastase present rather than its activity. These antigenic levels have not been studied often in GCF. The present study detected no statistically significant difference in either absolute amounts or concentrations of elastase in relation to inflammation or pocket depth. There

was, however, a tendancy for elastase levels to be higher at healthy and gingivitis sites. A possible mechanism for this will be proposed later.

Gustafsson et al (1992) examined the relationship of antigenic elastase and elastase activity in GCF using a substrate in two groups of patients; those with gingivitis and those with periodontitis. This study has the drawback of comparing two groups of patients rather than two types of sites within an individual. As a result it may be affected by the potentially large systemic variations which were to some extent avoided in the present study. In their reporting of differences between groups, the averages from two separate and unrelated sites were reported. They showed significantly higher concentrations and absolute amounts of elastase activity in patients with periodontitis. They did not however find any difference in either the absolute amount or concentration of antigenic elastase at the two types of site. In their study they attributed the lack of difference in antigenic elastase to the difficulties in relating clinical parameters to inflammation and assumed that elastase was related directly to the number of crevicular PMNs.

Meyle et al (1992) examined the amount of antigenic elastase in subjects following intensive oral hygiene and found no difference in the concentration of the complexed form of elastase i.e. that bound to alpha 1 antitrypsin, in relation to an improvement in oral hygiene. They did, however, find a significant difference in the amount of functional elastase, with a reduction during the improvement of oral hygiene. This they measured by adding pooled serum to the GCF samples. Unbound elastase was thus complexed with free alpha 1 antitrypsin in the serum and could be identified by the ELISA used. They reported functional elastase, proposing that it was free and unbound, as the difference between the complexed elastase in GCF and the total amount recorded after saturation with alpha 1 antitrypsin from serum. Using this method they concluded that antigenic and functional elastase activity in GCF did not follow the same pattern. No explanation for the lack of change in antigenic elastase was proposed, however they did speculate that the

increased level of functional elastase at inflamed sites was due to a deficiency of alpha 1 anti trypsin.

It is possible that in the present study, by measuring elastase in GCF using the ELISA technique we are recording differences similar to those observed by Gustafsson et al (1992) and Meyle et al (1992).

Alavi et al (1995) again attempted to address this discrepancy between functional and antigenic elastase at periodontal sites. They measured levels of antigenic and functional elastase in matched groups of smokers and non smokers. This study has the advantage of attempting to examine matched sites with regard to elastase activity and antigenicity and thus, if widely applied, overcomes the problems as outlined by Cox (1995). They collected GCF using Durapore filter strips, which enable the release and study of intact PMNs from the crevice. As a result they were able to perform GCF PMN counts in addition to the assays for antigenic and functional elastase. They found that the concentration of elastase in antigenic and functional forms was lower in smokers than in non smokers. They speculated that the increased level of tissue breakdown in smokers was not related to increases in elastase activity but may be attributed again to alterations in the binding of elastase to alpha 1 anti trypsin, as is seen in lung disease.

There appears to be a discrepancy between *in vivo* clinical findings with respect to elastase. Studies using elastase substrate assays provide evidence that elastase activity increases in GCF in gingivitis and at inflamed sites (Gianopoulou et al 1992, Darany et al 1992, Eley and Cox 1992, Smith et al 1995); in sites losing attachment (Palcanis et al 1992, Armitage et al 1994, Jin et al 1995); and that it decreases in response to basic, successful periodontal treatment (Cox and Eley 1992). These findings have been proposed as evidence for an increased release of elastase from PMNs at diseased sites. In none of these studies was the number of PMNs in GCF examined, nor was a cellular marker for

PMNs in GCF, such as lactoferrin, assayed. It was also assumed that functional elastase equated with that released from PMNs, Gustafsson et al (1992) and Meyle et al (1992) have shown that this may not be the case. It would seem logical that at sites of tissue destruction protease activity is higher, but it is possible that this is not due solely to an increase in the release of PMN granular proteases at these sites. The relative release of primary and secondary PMN granule components in the GCF samples was examined. The results support release of both primary and secondary PMN granule products into GCF at sites of periodontal health, gingivitis and periodontitis. In addition it is shown that a differential primary and secondary granule release occurs in healthy, gingivitis and periodontitis sites. There is a 10 fold relative increase in the amount of lactoferrin detected at periodontally involved sites. This difference may be accounted for by; 1) a relative decrease in the degranulation of primary granules, or 2) a relative increase in degranulation of secondary granules at periodontitis site.

The study detected no significant differences in either absolute amount or concentration of elastase at any category of site, indeed the amount of elastase appeared to be slightly higher at gingivitis sites than periodontitis sites. With regard to lactoferrin release, an increase in absolute amounts but not concentration was seen in periodontitis sites in comparison with gingivitis sites.

If release from primary and secondary granules is the same in all types of periodontal sites, it would be expected that the absolute amount of each granule component in GCF would increase in line with the volume of GCF and the number of PMNs, as is the case with lactoferrin. The finding that there is no increase in the absolute amount of elastase, despite the fact that the GCF volume and the number of PMNs are increased supports the former proposal i.e. that there is a relative decrease in the degranulation of primary granules at sites of periodontitis. This is in contrast to the findings of Gustafsson et al (1994) who proposed an exuberant degranulation of primary granules indicated by an increase in elastase activity relative to lactoferrin in inflamed crevices. Again this may be a

manifestation of the lack of a direct relationship between elastase like activity, measured by protease substrates in GCF, and the actual release of elastase from primary granules.

Alterations in PMN dynamics may be due to changes related to either phagocytosis or to the PMN response to immune complexes and immunoglobulins in the gingival crevice, as discussed in Chapter 1. Degranulation of PMNs generally occurs intracellularly into phagosomes, however there are mechanisms by which the contents are released extracellularly. These mechanisms are by cell death, perforation from within, regurgitation during feeding and reverse endocytosis (Goldstein 1984).

When release is by cell death this is generally as a consequence of cell damage by toxins or by injury. Damage to the plasma membrane causes the cell to rupture and release its contents into the surrounding medium. In such cases it would be expected to find equal proportions of all granule products as the whole cell is disrupted.

Perforation from within is a consequence of material in the interior of the cell directly rupturing lysosomal membranes. As cells die their contents are released extracellularly. Again, it would be expected that equal amounts of all markers would be present in this circumstance.

Regurgitation during feeding (Weissmann et al 1972) is the process whereby cells engaging in phagocytosis release some of their lysosomal products extracellularly from incompletely enclosed phagosomes. This is not accompanied by cell death and the cell remains viable. In this situation one would expect to find alterations in the content of the extracellular fluid in relation to the components involved in degranulation associated with phagocytosis

Finally, degranulation occurs without phagocytosis i.e. by reverse endocytosis. In this case neutrophils encountering immune complexes or immunoglobulins on solid surfaces adhere to the surface and release their granule contents directly into the extracellular environment (Goldstein 1976, Weissmann 1971).

Since PMNs collected from GCF are predominantly viable (Skapski and Lehner 1976, Kowolik and Raeburn 1980, Murray and Patters 1980, Charon et al 1982) the two mechanisms by which their granule contents are likely to be found in GCF are by regurgitation during feeding or by reverse endocytosis.

The degranulation of primary and secondary granules is under separate control (Henson 1971, Bentwood and Henson 1980, Murphy and Hart 1987,) with secretagogues effecting degranulation of either primary or secondary granules exclusively or preferentially (Boxer and Smolen 1988). Gustafsson et al (1994) reported degranulation assays using peripheral blood PMNs and opsonised *Staphylococcus aureus*. He confirmed the findings of others that PMN degranulation in respect of primary and secondary granules is separate in time and rate and proposed that in the gingival crevice PMNs were in an activated state at deep sites. However the degranulation assay which they used is not directly relevant to the situation in a periodontal pocket. *Staphylococcus aureus*, is not a micro-organism which is related to periodontal tissue destruction (Socransky and Haffajee 1992). In addition the bacteria were opsonised by human gamma globulin prior to incubation, as discussed below this mechanism may not be fully functional in relation to the gingival pocket. The cellular mechanisms which control these neutrophil secretions are only generally understood with G protein phosphorylation, protein kinase activity and changes in intracellular calcium being proposed (Fittschen and Henson 1994).

A decrease in degranulation of primary PMN granules has been described in several circumstances relating to infectious diseases. Evidence relates to the alteration in the

normal, separate control of degranulation effected by bacteria, viruses or infectious agent products. Quie (1983) reviewed inhibition of lysosome-phagosome fusion in relation to *Mycobacterium tuberculosis, Histoplasma capsulatum, toxiplasma gondii, Chlamydia* and some strains of *Neisseria gonorrheae*. This inhibition of degranulation occurs in the presence of normal PMN chemotaxis and phagocytosis.

Following phagocytosis of *Brucella abortus*, primary granule release is preferentially inhibited (Riley and Robertson 1984). In their study they showed that smooth and rough strains of *Brucella abortus* were both ingested normally but the smooth strain failed to stimulate primary degranulation after ingestion. This inhibition was related to a cell wall LPS. The result of failure to stimulate degranulation is decreased killing of ingested bacteria and increased pathogenicity. Bertram et al (1986) carried out further *in vitro* work on a *Brucella abortus* extract confirming inhibition of primary granule release and suggested that the inhibition was dose dependent relating to a nucleotide like material.

Inhibition of lysosome-phagosome fusion has been shown *in vitro* in relation to influenza virus and has been proposed as a factor in the increased susceptibility to bacterial infection coincident with influenza infection. Abramson et al (1982) showed that virus, or virus associated products, inhibit fusion of primary granules with phagosomes containing bacteria, the inhibition of that fusion correlates with decreased bacteriocidal activity.

Scrapie prion protein was shown to inhibit degranulation of primary granules of PMNs when in the native but not denatured form (Miragliotta 1989). They related this to a sialoglycoprotein macromolecule within the prion.

The differential release of granule constituents in relation to periodontally associated bacteria has not been much investigated. Baehni et al (1978) exposed peripheral blood

PMNs to supra and subgingival plaque and recorded the release of lysosomal markers. In addition they examined the ultrastructure of the PMNs during the experiment. They reported phagocytosis of micro-organisms and lysosomal degranulation. Their *in vitro* study showed an increased release of the lysosomal markers, lysozyme, B-glucuronidase, myeloperoxidase and lactoferrin in response to supragingival plaque compared with subgingival plaque.

Taichman et al (1977) exposed PMNs to irradiated plaque and recorded dose dependent PMN granule product release. The differences observed with viable plaque from supra and subgingval sites were absent. In all cases the addition of serum to the reaction mixture increased release from PMNs. On the basis of this serum dependant change they speculated that in the gingival crevice, PMNs release granule products by mechanisms that are not dependent on phagocytosis i.e. by reverse endocytosis mediated by complement or immunoglobulins. They further postulated that these mechanisms of degranulation are important in the extracellular killing of bacteria. This concept was supported by Wilton (1982) who proposed that complement fragments and immunoglobulins are important mediators of degranulation in the gingival crevice.

Although phagocytosis of plaque micro-organisms in the gingival crevice has been observed by many workers (Baehni et al 1978, Attstrom 1970) it is not the exclusive relationship of PMNs to micro-organisms. Tsai et al (1977) reported release of lysosomal enzymes in response to dental plaque which was independent of phagocytosis. Wilton (1986) suggested that phagocytosis was only likely for unattached fluid phase bacteria. Degranulation of PMNs was stimulated not only by phagocytosis but by other components of GCF, notably complement fractions C5a, C3b, C4b, immune complexes, bacterial toxins, IL-1 and by PMN migration. He proposed that since secondary granules were more abundant, and that release of their contents was more rapid and complete, it was possible that only these granules were released in the crevice.

The present study does not support only the release from secondary granules but does appear to show that at deeper, periodontally involved sites their release is not reduced in the manner of that from primary granules.

Taichman and McArthur (1976) proposed that not all periodontal bacteria stimulate lysosome release and related this ability to the synthesis of extracellular polysaccharides, as has been discussed in relation to other infectious agents above. Novak and Cohen (1991) reported a membrane depolarising agent associated with the capsule of some strains of *Porphyromonas gingivalis* which fails to initiate increases in intracellular calcium or to activate the respiratory burst. They suggested that this immobilises PMNs local activity and is an important virulence factor.

Thus from the evidence above it appears that if phagocytosis is not resisted bacteria may have an innate ability to suppress degranulation of primary granules. Alternately, co-incidental viral infection may affect degranulation. The results presented from this study may be an *in vivo* finding of the failure of periodontal pathogens to activate PMNs in the gingival crevice to produce reactive oxygen species and to degranulate.

When phagocytosis is not the most important mechanism for the control of micro-organisms, as is proposed in the gingival crevice, the alternative delivery of PMN primary granule contents is by reverse endocytosis. Reverse endocytosis is mediated by complememt and immune complexes binding to the surface of the PMN.

Arnold (1993) reported that even for patients with high antibody titres to *Porphyromonas gingivalis* antibody may not be opsonic and protease production by this bacteria may neutralise not only antibody but also complement. Kinane et al (1993) studied the level of IgG to P. *gingivalis* in periodontal pockets of different depths and

found that in deep pockets the level of antibody was reduced. Lopatin et al (1991) suggested that even in the presence of high antibody titres the avidity i.e. overall biological activity of the antibody in periodontitis sites may be low and thus contribute to the decreased elimination of bacteria and potentiate disease. Mooney et al (1993) studied a group of patients on a periodontal maintenance programme and compared the antibody titre and avidity in patients losing attachment and those not losing attachment over the study period. They showed that subjects losing attachment had lower baseline IgM avidity to *Porphyromonas gingivalis* than those who did not lose attachment. Cutler et al (1991) showed that effective binding of IgG to *Porphyromonas gingivalis* was essential in the opsonisation and phagocytosis of this organism and in complement activation.

In summary, it has been shown that local antibody levels are lower in deeper pockets and in inflamed sites than in shallower sites with less inflammation. Reverse endocytosis is dependent on these antibodies and complememt for opsonisation. Thus a reduction in the level or avidity of antibody or the destruction of complement by pathogens may be reflected by a decrease in primary granule degranulation and a relative weakening of the PMN defence in the area. There is the potential in the gingival crevice for phagocytosis and degranulation, either separately or together, to be disrupted, for local antibody to be ineffective due to its initial low titre and avidity in deep pockets and also for complement to be locally destroyed. In the face of the local paralysis of the PMN, bacteria have an increased opportunity to destroy tissue. With treatment the local bacterial challenge is reduced. This reduction encourages the production of antibody of higher avidity and biological function is improved. In addition complement is not degraded by bacterial proteases to the same extent and thus is more efficient in the opsonisation of bacteria.

A possible explanation of the present findings is as follows. In shallow pockets there may be bacteria that provoke complement activation, to which there is an appropriate immune response and which if removed by phagocytosis provoke degranulation of primary granules. In deeper pockets with inflammation where there is a reduced local antibody

level and antibody that is there is less effective in the activation of complement, less degranulation occurs. In addition those bacteria that are phagocytosed may not provoke the degranulation of primary granules. Thus the PMN response is reduced and bacteria can continue to function in tissue destruction.

4.4   Protease inhibitors in GCF

As previously discussed a fall in the relative degranulation from primary granules is in apparent conflict with the recorded increase in elastase activity in GCF at sites of inflammation reported by others. Alterations in the elastase activity recorded may be attributable to changes in binding of elastase to its major inhibitors, alpha 1 antitrypsin and alpha 2 macroglobulin. With alpha 1 antitrypsin protease inhibition is complete whereas with alpha 2 macroglobulin inhibition is incomplete. One possible explanation is that in disease states relatively more elastase is bound to alpha 2 macroglobulin than alpha 1 antitrypsin thereby increasing the residual protease activity of GCF.

Alpha 1 antitrypsin has been identified as of importance in clinical medicine (Kalsheker 1994) in relation to pulmonary disease and liver cirrhosis. It is proposed that a reduction in alpha 1 antitrypsin results in a loss of the antielastase shield in the lung and that this imbalance is the cause of early lung damage in individuals with alpha 1 antitrypsin deficiency. Like the lung the gingival crevice has a capillary bed with a tissue interface that is subject to bacterial and protease attack. Alavi et al (1995) proposed that alpha 1 antitrypsin may be of similar importance in the gingival crevice. However in the present study the absolute amount of alpha 1 antitrypsin was significantly increased not decreased at gingivitis and periodontitis sites relative to healthy sites. No significant differences were recorded in relation to its concentration.

Alpha 1 antitrypsin may have other functions in GCF. Various concentrations have been examined for the ability to promote PMN chemotaxis (Aoshiba et al 1993). They showed that the inhibitor was capable of causing *in vitro* chemotaxis and chemokinesis at concentrations of 0.02mg/mL, 0.2mg/mL and 2mg/mL, with maximum potency at 2mg/mL. Above that level, up to 10mg/mL, chemotaxis of PMNs in response to known chemoattractants was inhibited. They hypothesised that alpha 1 anti trypsin had a regulatory role on PMN recruitment at sites of inflammation whereby at 0.2mg/mL the concentration found in alveolar fluid in the lung PMNs were attracted to protect the airways. The 2mg/mL in blood was sufficient to prevent excessive loss of PMNs from the circulation.

The mean concentration of alpha 1 anti trypsin at healthy sites was 2615ng/ul (range 35-10176, SD 3616), at gingivitis sites 4993ng/ul (range 941-20774, SD 5766) and at periodontitis sites 3469ng/ul (range 678-19362, SD 5639). This equates to 2.615mg/mL, 4.993mg/mL and 3.469mg/mL respectively. Bearing in mind the inaccuracies in recording concentrations of GCF components and the fact that these average values are higher by a factor of ten than was recorded by Adonogianaki et al (1992), concentrations of alpha 1 antitrypsin found in the gingival crevice probably fall into the range in which chemotaxis and chemokinesis is promoted. The level of correlation of alpha 1 antitrypsin with elastase and lactoferrin further support this possible mechanism in the gingival crevice. If this system is operational alpha 1 antitrypsin may play a crucial role in the control of PMN movement towards the gingival crevice.

In relation to alpha 2 macroglobulin in GCF the absolute amount recovered rose sharply with inflammation and was significantly higher in gingivitis and periodontitis sites relative to healthy. With regard to the concentration of alpha 2 macroglobulin, this rose at gingivitis sites which were significantly different from healthy sites but not from periodontitis sites.

Sengupta et al (1988) found lower levels of alpha 2 macroglobulin after the improvement of clinical parameters. In contrast Skaleric et al (1986) found lower levels of alpha 2 macroglobulin at sites with a history of alveolar bone loss and with inflammation. This difference may be due to collection and reporting methods. They collected GCF of a predetermined volume using microcapillary tubes, this is traumatic to tissues (Cimasoni 1983) and causes dilution of the components in the crevice as a result of increasing vascular permeability. In addition all values were expressed in concentrations which again may be less reliable means of presentation for samples (Lamster et al 1986).

Studies examining both inhibitors in the same sample tend to have similar findings. Levels recorded in the present study follow the pattern previously described using the same assay with GCF in a cross sectional trial (Adonogianaki et al 1992). They found higher absolute amounts of alpha 1 antitrypsin and alpha 2 macroglobulin at inflamed and deep sites compared with healthy sites. In relation to alpha 1 antitrypsin the current levels are higher than previously recorded. This increase is likely due to the very wide range of values recorded for alpha 1 antitrypsin and the relatively small number of samples in the present study.

In their study of healthy and periodontally involved teeth Ohlsson et al (1974) found alpha 1 antitrypsin and alpha 2 macroglobulin in the gingival crevice at sites of both health and disease. They reported that the level of both inhibitors was higher at sites of inflammation and that alpha 1 antitrypsin was saturated with respect to its protease inhibiting capacity. Adonogianaki et al (1994) demonstrated an increase in the inhibitors alpha 1 antitrypsin and alpha 2 macroglobulin over a 21 day experimental gingivitis period. They attributed this to the increase in serum derived inhibitor and also an increase in the local production of alpha 2 macroglobulin from fibroblasts, alpha 1 antitrypsin and alpha 2 macroglobulin from monocytes and macrophages and alpha 1 antitrypsin from PMNs.

In experimental gingivitis trials what is being examined is change in site characteristics when comparing sites with no inflammation and sites with inflammation. What is ultimately clinically useful is to differentiate between sites which are periodontally active and those which are not. In a prospective study addressing this issue Adonogianaki et al (1996) examined the ability of the acute phase proteins, including alpha 1 antitrypsin, alpha 2 macroglobulin and lactoferrin, to identify active and stable sites in periodontal maintenance patients. They found that the 3 markers could not discriminate between sites at baseline on the basis of attachment loss. Probing attachment loss was not preceded by a change in these markers and they proposed that they were all markers of gingival inflammation rather than destruction. In addition they proposed that exuberant PMN activity, if measured by lactoferrin, was not a feature of periodontally active sites.

Thus, the present study is in line with others in finding alpha 1 antitrypsin and alpha 2 macroglobulin in GCF. It is also in agreement that the level of both inhibitors increases at sites of inflammation. In addition it confirms other work that neither the absolute amount or concentration of either of these inhibitors can differentiate gingivitis and periodontitis sites (Adonogianaki et al 1992).

The present study showed a tendency for the level of alpha 1 antitrypsin relative to alpha 2 macroglobulin to be increased at healthy and periodontitis sites compared with gingivitis sites, this difference did not reach significance. There was a high correlation between alpha 1 antitrypsin and alpha 2 macroglobulin. Thus a shift in the importance of either the complete inhibition of elastase, alpha 1 antitrypsin, at sites of health to increased relative inhibition by alpha 2 macroglobulin at diseased sites is not supported. In addition the pattern of change in inhibitors from one site to another was similar in 7 of the 10 subjects from whom GCF was collected.

Protease inhibitors in the GCF are derived from two sources; either from the serum or local cells among which are fibroblasts, monocytes, macrophages and PMNs. Condacci et al (1988) demonstrated the *in vitro* ability of gingival fibroblasts to synthesise alpha 2 macroglobulin. In a further clinical study the group examined the relationship of alpha 2 macroglobulin to albumin during experimentally induced gingivitis. In the study albumin was used as a marker of serum derived protein. They found that the concentration of albumin increased two fold during the experimental period and the concentration of alpha 2 macroglobulin by around six fold. They attributed this increase in alpha 2 macroglobulin to both increased passage of inhibitor from the blood vessels and local production.

Since the local sources of inhibitors are different and since the pattern of change observed is similar in the majority of cases, it would seem unlikely that *in vivo* local production of inhibitors is contributing greatly to the GCF pool. This is in agreement with the proposals of Lamster et al (1990) and Adonogianaki et al (1996). Alternatively local production of inhibitors may be largely confined to and important in, the connective tissue interface.

4.5   Elastase and Protease inhibitors in GCF

The level of alpha 1 antitrypsin relative to elastase increased significantly from healthy to gingivitis and periodontitis sites. This does not support the idea that increased elastase activity is due to a lack of alpha 1 antitrypsin or a shift away from effective protease inhibition as is seen in the lung. It also further reinforces the host attempt at protection in deep and inflamed pockets and may be a manifestation of the local production of inhibitor in the increased number of PMNs.

du Bois et al (1991) recently demonstrated expression by PMNs of the gene for alpha 1 antitrypsin and confirmed the ability of the cells to produce the inhibitor. This gives

neutrophils the potential to protect the local environment from the damaging effects of their secreted proteases, although the contribution from this source to body fluids is still thought to be less than that from hepatocytes and monocytes. They postulated that the role of locally, PMN produced, alpha 1 antitrypsin was in the inhibition of elastase from PMNs adherent to surfaces where systemically derived inhibitors are thought to have little effect. They further postulated that in circumstances where neutrophils are not releasing major amounts of elastase this mechanism may be efficient in the inhibition. However, where large amounts of elastase are released or at fluid interfaces oxidants produced by the neutrophils and the absolute amount of released protease would overcome the defence. They suggested that this mechanism protects host tissue from protease degradation during PMN migration through tissues. This may be a mechanism operational in healthy gingival crevices and tissue where elastase is found, but where it appears that there is effective inhibition of tissue destruction.

Alpha 1 antitrypsin inactivation occurs in the presence of oxidants (Weiss 1989) and has recently been shown to be caused also by metallo-proteinases in myeloperoxidase positive granules (Ottonello et al 1994). Although data in the study is not available regarding the state of activity of either elastase or its inhibitors, a possible explanation for the increase found in elastase activity despite a relative decrease in the degranulation of PMNs may be as follows. At deeper sites with inflammation there may be more inactivation of the inhibitor by oxidants generated in the respiratory burst or by other granule components. Alternatively the local source of alpha 1 antitrypsin as described by du Bois may be overcome thus enabling increased local protease activity.

With reference to alpha 2 macroglobulin relative to elastase levels rose from health through to periodontitis. Significant differences were observed between shallow non inflamed sites and inflamed sites but not shallow and deep inflamed sites i.e. gingivitis and periodontitis sites. One possibility, that the amount of alpha 2 macroglobulin increases thus causing incomplete protease inhibition at deep sites with active destruction, is not supported. In

addition the increase in the level of inhibitor relative to protease again suggests an attempt to protect host tissues.

Using a patient profile similar to that reported previously, Gustafsson et al (1994) studied elastase activity and alpha 2 macroglobulin in GCF of gingivitis and periodontitis patients. They reported higher amounts of elastase activity and lower alpha 2 macroglobulin levels at sites with destruction compared with those with only superficial inflammation. This is in contrast to the findings with regard to the present study which detected higher levels of inhibitor and a higher ratio of inhibitor to antigenic elastase at inflamed sites. Again they proposed that this was attributed to increased numbers of crevicular PMNs and that these were in an activated state. They proposed that lower levels of alpha 2 macroglobulin were found because of its increased consumption by proteases and its subsequent uptake in the reticulo-endothelial system. It may be that in this study by the reporting of concentrations they are incorporating differences related to the increased volume of GCF at deep sites in relation to alpha 2 macroglobulin.

Giannopoulou et al (1992) in the experimental gingivitis study previously described found that both the total amount and concentration of inhibitors rose significantly during the development of inflammation. Their assays demonstrated that antigenic elastase in the GCF eluted with alpha 1 antitrypsin and functional elastase with alpha 2 macroglobulin. The present study concurs with their finding of a rise in elastase and its inhibitors at sites of inflammation.

As discussed previously many workers use fluorogenic substrates for the evaluation of elastase in GCF and equate elastase activity with the function of primary granules. Using these substrates they propose that there is an increase in the release of elastase from primary granules at sites of periodontal disease. In view of the elution of elastase with alpha 2 macroglobulin this may not be a valid assumption i.e. elastase activity is not due

to free elastase. Instead, the activity of the elastase released by primary granules may be modified by other factors in the GCF thus accounting for the discrepancy between elastase and elastase like activity changes.

A study reported by Rosin et al (1995) supports this proposal. They studied the level of complexed alpha 2 macroglobulin and the total amount of alpha 2 macroglobulin in shallow and deep periodontal pockets. They showed no change in concentration of these two forms between shallow and deep sites. They found that the total alpha 2 macroglobulin was related to protease activity in GCF and inflammation and that at all sites around 70% of alpha 2 macroglobulin was present in the complexed form, with no difference between shallow and deep sites. They showed no correlation between the complexed form of inhibitor and protease activity at shallow sites. They suggested that the amount of complexed inhibitor was related not only to the protease load in GCF but also to the conversion of alpha 2 macroglobulin by bacterial end products, the presence of matrix metalloproteinases and complexed inhibitor entering the GCF as a result of normal tissue turnover.

4.6 Conclusions

1 The previous findings of Adonogianaki et al (1993) that lactoferrin correlates with pocket depth and GCF volume are supported.

2 It is proposed that the independent measurement of elastase activity may not be an indicator of crevicular PMN function.

3 The alteration in the relationship of the primary PMN granule marker elastase and the secondary granule marker lactoferrin may be of clinical use in the differentiation of gingivitis and periodontitis.

4 A mechanism involving the local paralysis of PMN function in deep pockets is proposed. This would effectively disable the cells and prevent them from releasing granule contents which may protect the host by eliminating destructive bacteria.

5 The levels of protease inhibitors alpha 1 antitrypsin and alpha 2 macroglobulin were not reduced at sites of periodontitis. The levels of these 2 inhibitors had a high correlation, r=0.861. This would tend to support the principle source of these inhibitors in GCF being the same.

6 The increased breakdown in relation to periodontitis sites does not appear to be related to an alteration of the relative importance of either of the proteases measured in GCF.

4.7 Future Work

The study presented could be viewed in the nature as pilot study. The most interesting aspect of this work is the difference in the level of degranulation for primary and secondary PMN granules at deep, inflamed sites. In view of the poor recovery of elastase from samples further studies need to incorporate:

- a larger number of subjects

- more than one area representing each type of site.

- an alternative PMN primary granule marker e.g. Beta glucuronidase, or attempt to refine a sandwich ELISA for PMN elastase.

- an assessment of functional and antigenic elastase and inhibitor complexes

Together these would provide more power to observe differences suggested by the pilot study.

In addition, it would be interesting to combine further study of degranulation dynamics with a study of the bacterial flora at the sampled sites. In the present study, the microbiology of sites was not assessed. Assumptions regarding the presence of particular periodontal pathogens at deep sites were made, particularly in relation to *Porphyromonas gingivalis*. In addition the continued study of inhibition of opsonisation and degranulation in relation to periodontal pathogens may give insight into the defence mechanisms operating in the gingival crevice.

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EPILOGUE

The Lotos blooms below the barren peak:

The Lotos blows by every winding creek:

All day the wind breathes low with mellower tone:

Thro every hollow cave and alley lone

Round and round the spicy downs the yellow Lotos-dust is blown.

We have had enough of action and of motion we,

Roll'd to starboard, roll'd to larboard, when the surge was seething free,

Where the wallowing monster spouted his foam-fountains in the sea.

Let us swear an oath, and keep it with an equal mind,

In the hollow Lotos-land to live and lie reclined

On the hills like Gods together, careless of mankind.

For they lie beside their nectar, and the bolts are hurl'd

Far below them in the valleys, and the clouds are lightly curl'd

Round their houses, girdled with the gleaming world:

Where they smile in secret, looking over wasted lands,

Blight and famine, plague and earthquake, roaring deeps and fiery sands,

Clanging fights, and flaming towns, and sinking ships, and praying hands.

But they smile, they find a music centred in a doleful song

Steaming up, a lamentation and an ancient tale of wrong,

Like a tale of little meaning tho' the words are strong;

Chanted from an ill-used race of men that cleave the soil,

Sow the seed, and reap the harvest with enduring toil,

Storing yearly little dues of wheat, and wine and oil;

Till they perish and they suffer - some, 'tis whispered-down in hell

Suffer endless anguish, others in Elysian valleys dwell,

Resting weary limbs at last on beds of asphodel.

Surely, surely slumber is more sweet than toil, the shore

Than labour in deep mid-ocean, wind and wave and oar;

Oh rest ye, brother mariners, we will not wander more.

<div align="right">

Tennyson

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