MOLECULAR AND CELLULAR

ASPECTS OF THE HUMORAL IMMUNE RESPONSE

IN PERIODONTAL DISEASES AND OTHER RELATED CONDITIONS

By

John Mooney B.Sc., M.Sc. (Glasgow)

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

Department of Oral Sciences, University of Glasgow Dental School

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To my dearest Laura,
with all my love

DECLARATION

This thesis is the original work of the author.

John Mooney

Chapter 1

Introduction

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Acknowledgements

I should like to thank everyone who provided me with advice and encouragement during the course of this project. Deserving of particular mention are my supervisor, Professor Denis Kinane for his enthusiasm; the Dean of the Dental School, Professor Wallace MacFarlane, for his advice; Dr Eva Adonogianaki, Mrs Maura Edwards, Dr Keiso Takahashi and Dr Ahmed Haerian for the collection of clinical samples; and, most importantly, my wife, Laura, for her continued support during a further post-graduate degree.

List of Abbreviations

AP: adult periodontitis

ATCC: American Type Culture Collection

AUG or ANUG: acute (necrotising) ulcerative gingivitis

BCIP: bromochloro-indoylphosphate

BOP: bleeding upon probing

BSA: bovine serum albumin

CFW: crevicular fluid washings

DEPC: diethylpyrocarbonate

ELISA: enzyme-linked immunosorbent assay

EOP: early-onset periodontitis

FAB: fastidious anaerobe broth

GALT: gut-associated lymphoid tissue

GCF: gingival crevicular fluid

GJP: generalised juvenile periodontitis

HPT: hygiene-phase therapy

ISH: *in situ* hybridisation

JP: juvenile periodontitis

LJP: localised juvenile periodontitis

LPS: lipopolysaccharide

MGI: modified gingival index

MHC: major histocompatibility complex

NBT: nitroblue tetrazolium

NCTC: National Collection of Type Cultures

NSAIDs: non-steroidal anti-inflammatory drugs

PAF: platelet activating factor

PBS: phosphate-buffered saline

PBST: phosphate buffered saline Tween

PBSTM: phosphate buffered saline Tween with 5% non-fat dried milk

PCR: polymerase chain reaction

PD: pocket depth

PDI: periodontal disease index

PI: plaque index

PISF: peri-implant sulcular fluid

PMN: polymorphonuclear leucocyte

RA: rheumatoid arthritis

RCE: relative coefficient of excretion

RM: recurrent miscarriage (or abortion)

RPP: rapidly progressive periodontitis

SSC: standard saline citrate

TE: Tris EDTA (ethylenediaminetetraacetic acid)

TIMP: tissue inhibitor of matrix metalloproteinases

TPO: thyroid peroxidase

Summary

The aims of this study fall under four broad headings. The first was to follow up previous work in this laboratory which had indicated that local disease status is related to levels of specific immunoglobulin. A previous study had shown that gingivitis and periodontitis sites can be distinguished in that the latter have lower antibody levels to *P. gingivalis* in the GCF than the former, perhaps indicating local degradation by oral pathogens. In the present study, the experimental design was modified to allow matching of gingivitis and periodontitis sites within the same patient, generating the potential for a more powerful statistical analysis.

Reports demonstrating differences in specific humoral immunity with ageing prompted an experimental gingivitis study on periodontally healthy young and old subjects, to investigate possible differences in local immune and inflammmatory parameters. Since chronic periodontal disease is primarily a disease of ageing, any immune deficiencies contributing to periodontal disease may be related to the ageing process itself.

A previous report from this laboratory had suggested that there may be differences in immune and inflammatory

processes at matched oral implant and natural tooth sites in the same patient. Part of this study, therefore, was to follow up these investigations.

The second aim was to investigate local production of immunoglobulins by plasma cells in the gingiva. Therefore, separate investigations quantified the proportions of κ and λ light-chain producing plasma cells and also IgG and IgA subclass-producing plasma cells in the gingiva and related these to levels of the immunoglobulins in the GCF.

Previous work in this laboratory has demonstrated the significance of specific serum antibody avidity to periodontal diagnosis and prognosis. Therefore, the third aim of this study was to investigate the impact of antibody avidity on the outcome of periodontal treatment. A preliminary study was set up to assess these effects; also attempting to relate microbiological parameters. A larger study was then conducted in which patients were assigned to seropositivity groups at baseline. They were then followed longitudinally to assess differences in treatment outcome.

A fourth aim of this study was to look at the wider significance of immunological parameters in disease pathology. Both periodontal disease and pregnancy can in some ways be seen as a model immune-inflammatory processes.

Therefore, the relevance of serum levels of IgG subclasses to pregnancy outcome was assessed.

Thus the overall aim of this project was to increase understanding of the immunological aetiology of periodontal disease at local and systemic, cellular and molecular levels; and to put these findings into the wider context of immune-inflammatory processes.

Confirmation of the immunological difference between gingivitis and periodontitis sites was achieved. Similarly, local humoral immune and inflammatory reactions were found to differ in older and younger individuals in that the former had lower IgG1, IgG4 and lactoferrin levels than the latter. Rates of local antibody production for matched teeth and implants were found not to correlate suggesting differences in the plasma cell infiltrate.

A predominance of κ light chain positive cells was found among plasma cells in inflamed gingival tissues. The κ/λ ratio of inflamed sites could be a useful method of distinguishing the stage or activity of periodontitis lesions and may differ between different forms of periodontitis. The levels of IgG subclasses in the gingival crevicular fluid were found to correlate with levels of production by local plasma cells. However, levels of local IgA1 were much lower than plasma cell

numbers would indicate, suggesting degradation by bacterial proteases. This was further indicated by high levels of IgA1 F_{ab} fragments.

The studies of periodontal treatment effects indicated that treatment outcome was predicated on initial serostatus to periodontal pathogens, and that increases in antibody avidity only occurred in patients who were initially seropositive.

The studies of obstetric immunology showed that levels of IgG subclasses were significantly increased during the first trimester of normal pregnancy, but this increase did not occur in women who had spontaneous abortion.

In conclusion, this project has confirmed the findings of earlier work in this laboratory, produced new findings on ageing effects, treatment effects and plasma cell infiltration in periodontal disease, elucidated further the immunology of pregnancy, and established areas for further research.

Chapter 1

Introduction

1. Introduction

1.1 Periodontal disease: Prevalence, causes and host specificity

1.1.1 Introduction

Periodontal disease is a general term which can be used to refer to all diseases which could have an effect on the periodontium. Since the periodontium comprises tissues of different origin, both mesenchymal and ectodermal, a large range of systemic conditions with periodontal consequences could be included under this heading (1). However, in the present context, this term will only be used to denote gingivitis and periodontitis, i.e. dental plaque-induced inflammatory processes of the periodontium.

1.1.2 Gingivitis

In gingivitis, pathological changes are confined to the superficial gingival tissue, i.e. the gingiva. Clinical manifestations include redness and swelling, and an increased tendency to bleeding on probing, all associated with increased vascularity. The increased vascularity also leads to an increase in local temperature and

increased gingival crevicular fluid flow. Gingival crevicular fluid (GCF) is an inflammatory exudate or preinflammatory transudate present in the gingival crevice. Its components are derived mainly from: 1) microbial products; 2) interstitial fluid and locally-produced factors of host origin; 3) plasma; and 4) tissue degradation/turnover products (2). Production of GCF due to an inflammatory increase in the vascular permeability of the subendothelial vasculature was shown in early studies (3, 4).

1.1.3 Periodontitis

Periodontitis, however, also effects the deeper structures of the periodontium, i.e. cementum, periodontal ligament and bone attachment. This results in loss of periodontal support, bone loss as evidenced by radiographs and the development of the periodontal pocket. At least four different forms of periodontitis have been defined (5-7), and, as will be discussed later, it is thought that at least some of these have an aetiological basis related to differences in their host response.

Early studies in this field (8-10) contributed to the consensus that: 1) periodontitis is a virtually universal phenomenon affecting a very high percentage of the population; 2) the disease begins as gingivitis at an early age and if left untreated will develop into periodontitis; and 3) most of the perceived variance in periodontal disease is associated with age and deficient oral hygiene.

1.1.4 Current Concepts in Epidemiology

However, recent studies have challenged these concepts and a different overall picture of the prevalence and progression of destructive periodontal disease has emerged.

Recent cross-sectional studies have demonstrated that, although a periodontium of reduced height becomes the norm with increasing age, relatively few subjects in each age group suffer from advanced periodontal destruction. These subjects account for most of the sites which are severely periodontally involved (11-13). Papapanou *et al* reported that 75% of the total sites with

previous attachment loss of at least 6mm occurred in 23% of the individuals examined (12).

Loe *et al* (14) reported similar findings in a 15-year-long study of the natural history of periodontal disease in Sri Lanka. These investigations identified a group of subjects, comprising 8% of the total sample, who showed rapid progression of periodontal disease, as defined by tooth mortality rates and interproximal attachment levels. In the same study, however, 11% of the subjects did not show progression of periodontal disease beyond gingivitis.

In addition, longitudinal studies have shown that relatively few sites undergo extensive periodontal destruction within a given observation period (15-17). Lindhe *et al* (16) reported that during a six year follow-up of 64 Swedish subjects, showing signs of previous periodontal destruction but not receiving active periodontal treatment, only 3.9% and 11.6% of their sites demonstrated attachment loss of greater than 2mm from baseline at 3 and 6 years respectively. Similar percentages (3.2%) of sites exhibiting periodontal attachment loss were reported in the same study for a group of American subjects who were followed over a one

year period. Moreover, it seems that relatively few individuals account for the sites which show active periodontal destruction over an observation period. Thus, Lindhe *et al* (17) in a recent study reported that 70% of the sites that deteriorated by 3mm or more during a two year monitoring period occurred in only 12% of the 265 Japanese subjects that were followed longitudinally.

1.1.5 Subject and Site Relatedness

Therefore, the current view is that periodontal disease is subject related, with a small subset of individuals within a given population suffering from advanced periodontal destruction, and with relatively few subjects and sites undergoing active periodontal destruction within a given period. In addition, in the absence of treatment, the mere presence of inflammatory periodontal lesions does not necessitate their progression (18). Thus, it has become apparent that any investigation of periodontal disease must take account of subject-specific and site-specific factors, and this will become clearer in the later discussion of subject and site susceptibility.

1.1.6 Recent advances in epidemiological understanding

Recent reports have added to our understanding of the epidemiology of periodontal disease. Kerr has shown that, in an archaeological study of an English population dating from 1645-1852 i.e. in a natural state without dental care, the prevalence and distribution of periodontitis lesions were similar to those in modern English populations (19). The study confirmed the existence of susceptible and resistant subgroups of individuals and of teeth. However, the author concludes that the findings do not support the view that periodontal disease is the primary cause of tooth loss in the untreated population. It must be borne in mind that this is a controversial conclusion which does not seem to take account of rapidly progressive and site-specific forms of periodontal disease, susceptible individuals and sites, and the apparently higher rates of periodontal destruction among the much older groups surviving in modern populations.

For example, Fox et al (20) conducted a study of over 500 people in New England between the ages of 70 and 96.

This New England Elders Dental Study (NEEDS) revealed substantially higher estimates of periodontal destruction among older adults than previous U.S. studies seemed to suggest. They conclude that the increasingly ageing population presents a challenge for periodontal treatment provision.

Bagramian et al (21) studied a large number of Amish and non-Amish people in Michigan. The Amish reject many elements of modern lifestyle and their rate of daily toothbrushing is much lower (37%) than in non-Amish individuals (85%). Most periodontal measurements seemed to be poorer in the Amish individuals, but these differences were not significant. However, this report is complicated by the inter-relatedness of lifestyle factors and genetic similarity in the Amish group, which is genetically more homogeneous than the general population.

Genco (22) has assessed risk factors for periodontal disease, and has found smoking and diabetes to be important systemic risk factors. Important local periodontal pathogens were found to be *Porphyromonas gingivalis* and *Bacteroides forsythus*. Watson et al (23) used the ability of *Porphyromonas gingivalis, Treponema denticola* and *Bacteroides forsythus* to hydrolyse the

synthetic trypsin substrate benzoyl-DC-arginine napthylamide (BANA) to demonstrate that children whose parents were colonised by these BANA-positive bacteria were 10 times more likely to be infected themselves than children whose parents were not. They conclude that their data are compatible with the hypothesis that children may acquire these species from their parents, especially if the parents have periodontitis. Therefore, transmission of pathogenic organisms within the family group may be an important factor.

The microbiology of periodontal disease is discussed further in section 1.2., and other factors and diseases relevant to periodontal disease are discussed further in section 1.4.

Grossi et al (24,25) have confirmed the importance of age, smoking, diabetes mellitus and subgingival colonisation by *Porphyromonas gingivalis* and *Bacteroides forsythus* as risk factors for attachment loss. The occurrence of low levels of periodontal disease in untreated people in underdeveloped countries has been confirmed in West Bengal by Maity et al (26). Moreover, Diamanti-Kipioti et al (27) have recently confirmed the existence of a skewed distribution of advanced disease in

the population. Their study on a Greek population showed that 25% of the rural sample and 12% of the urban sample accounted for 75% of the total number of tooth sites with pronounced bone loss. Loesche has demonstrated that periodontal disease is a risk factor for heart disease (28) and it has previously been shown that there is a link between periodontal disease, heart disease, smoking and socio-economic group (29). This will be further discussed in section 1.4.

1.1.7 The Classical Model

Page and Schroeder (30) proposed five distinct states in the pathogenesis of periodontitis, ranging from health to advanced disease. The stages within this classical picture are health, the initial lesion, the early lesion, the established lesion and the advanced lesion. The initial PMN infiltrate was postulated to be rapidly replaced within 48 hours by a lymphocytic lesion. This lesion could then stabilise as the early lesion unless

further challenged by plaque. With further plaque challenge, however, the cellular infiltrate was then thought to transform into a plasma cell dominated lesion. At this stage tissue destruction including bone resorption was thought to occur.

However, three factors have brought Page and Schroeder's model into serious question: 1)the inability to test the validity of the model with true longitudinal studies; 2)recent changes in ideas on periodontal disease activity; and 3) the fact that not all postulated stages have been observed histologically (31-35).

1.1.8 Three Models of Pathogenesis

Three models have been described to explain destruction of the periodontal supporting tissues: the continuous paradigm, the random burst theory and the asynchronous multiple burst hypothesis. The continuous paradigm postulates slow, constant and progressive destruction, and is supported by cross-sectional studies (36,37), and

longitudinal monitoring of sites not responsive to treatment (38). However, as the study of Loe *et al* (37) shows, pooling data from groups, individuals and sites in cross-sectional studies can give the impression that destruction was slow and continuous.

The random burst theory proposes short periods of destruction punctuated by periods of resolution occurring randomly in time and at random sites within the subject (39). By contrast, the asynchronous multiple burst hypothesis proposes that destruction occurs within a defined time frame and then resolution or remission follows. This hypothesis suggests that many sites would show bursts of activity over a limited period of time and then these sites would become inactive indefinitely. None of these proposed mechanisms can be established or refuted by presently available data (40). However, a matter of some concern must be that methods of assessing parameters, such as attachment loss, generally provide poor resolution. This means that such parameters will, almost by definition, lend themselves to the detection of "bursts" during longitudinal trials.

1.1.9 Stages in Pathogenesis

The Page and Schroeder model will be used as a framework for the following discussion of each stage in the pathogenesis of periodontal disease, but conflicting views will also be presented where appropriate.

Under experimental conditions, clinically "healthy" gingiva can be established. The tissues are characterised by gingival index (GI) scores of 0 and a mere trace of gingival exudate (41, 42), but this is not identical with histologically "normal" gingiva (43). This normal condition only exists in gingiva adjacent to plaque-free teeth and is, therefore, rarely encountered in humans. It has been described in animals whose gingivae have been kept meticulously clean (44) and also in germ-free animals (45). It must be stressed that even biopsies of clinically normal human gingiva demonstrate infiltration of inflammatory cells. This infiltrate comprises 3-5% of the connective tissue volume contiguous with the junctional epithelium, and contains PMNs, monocytes, macrophages and lymphocytes, primarily T-cells with very few B-cells or plasma cells (30,34,46). This infiltrated area suffers from collagen depletion and its vascularity is increased.

Fluid and plasma proteins leak from the microvasculature, percolating through the connective tissue and junctional epithelium into the gingival sulcus, giving rise to the GCF (47).

This stage contributes one of the main problems in elucidating the pathogenesis of periodontitis. Investigators have been unable to make a clear distinction between normal and pathologically altered tissue. The crucial point at which disease commences has not been determined. Since definitive evidence on this point is lacking so far, two hypotheses have been adduced. The first states that the features of the initial lesion merely reflect enhanced levels of the host defences occurring within the gingiva as part of normal surveillance procedures (30,32,48,49). The second states that the "initial" stage may be a quiescent phase following destructive disease. As such it may constitute a later or indefinitely recurring stage in disease progression (50).

The acute, exudative, inflammatory response to plaque accumulation is known as the initial lesion (32,49), according to the Page and Schroeder classification. The initial lesion usually manifests after 2-4 days, although

vascular changes beneath the junctional epithelium can be seen within 24 hours of plaque accumulation; it is localised around the gingival sulcus. More blood is brought to the area by dilation of the arterioles, venules and capillaries of the dentogingival plexus. At the same time, intercellular spaces between capillary or venular endothelial cells appear because of the elevation of hydrostatic pressure. This causes increased permeability of the microvasculature resulting in leakage of fluids and proteins into the tissues. The result is the GCF flow which may act as a defence mechanism by flushing out bacterial products while, at the same time, delivering bactericidal host products, e.g. antibodies and complement.

Listgarten and Ellegaard (51) have shown that plasma cells predominate apically to the gingival sulcus in the initial lesion in animal models. However, in human models, the initial lesion contains no plasma cells in the connective tissue area subjacent to the junctional epithelium (32,52). It has been suggested that host defence mechanisms may not always have a beneficial role, and that they may also be destructive. Although neutrophils and macrophages are recruited to the area to

defend the host against bacterial attack, their accumulation in the coronal part of the connective tissue and junctional epithelium probably accounts for much of the damage seen in this portion of the gingiva (53-56).

The initial lesion transforms into the early lesion within about one week of plaque accumulation (32,57). The vessels of the coronal portion of the dentogingival plexus remain dilated (44,47), and the additional plaque accumulation results in more pronounced infiltration of the dentogingival epithelium by PMNs and macrophages. The inflammatory cell infiltrate now occupies 10-15% of the connective tissue volume of the free gingiva (57) and contains T-cells and some B-cells (34). Lymphocytes predominate in the early lesion but few plasma cells have been demonstrated (32,34,35,51).

The basal cells of the junctional/sulcus epithelium proliferate at this stage, and rete pegs have been demonstrated invading the coronal portion of the infiltrate (58,59). The character of the cellular infiltrate and the nature of the pathological alterations observed have led to the concept that cellular hypersensitivity may be an important aspect of the early lesion. Wilde, Cooper and Page (60) demonstrated that

typical early lesions could be created in the gingival tissue of rats and monkeys sensitised to skin contact antigens followed by challenge at the gingival margin with the same antigen. They suggested a specific T-cell-mediated mechanism, since sensitisation can only be transferred to unsensitised animals by means of lymphocytes, but not serum.

The duration of the early lesion has not been determined. Recently, a six-month experimental gingivitis study by Brecx *et al* (52) has demonstrated that more than six months of oral hygiene abstention is required before plasma cells dominate the lesion. Therefore, the early lesion may continue for a much longer period than previously thought.

The established lesion, as defined by Page and Schroeder, is one dominated by plasma cells. Many investigators have stated that only 3-4 weeks of plaque accumulation are required for the formation of a plasma cell dominated lesion (32,61). However, this assumption has been challenged by Brecx *et al* (52).

The established lesion continues to exhibit the features of the preceding stages, but to a more marked degree (59,62). Large numbers of plasma cells are seen

(59,63), which are primarily located in the coronal connective tissue and around vessels in more distal gingival connective tissue. Collagen loss continues in both apical and lateral directions as the inflammatory cell infiltrate expands, resulting in collagen-depleted areas radiating deeper into the tissues (54).

Two types of established lesion appear to exist, some remaining stable and not progressing for months or years (64-67), while others become more active and convert to progressive destructive lesions. Controversy surrounds the nature of this conversion. Seymour, Powell and Davis (68) hypothesised that a change from T-cell to B-cell dominance presages the conversion from stability to activity involving aggressive destruction. However, Page (69) has disagreed with this view; a recent study has shown B-cell infiltrate mainly associated with stable, non-progressive lesions in childhood gingivitis (70).

The final stage in this process is known as the advanced lesion. The advanced lesion has characteristic features including alveolar bone loss and fibrosis of the gingiva with widespread manifestations of inflammatory and immunopathological tissue damage (71,72). The lesion is no longer localised and the inflammatory cell infiltrate

extends laterally and apically into the connective tissue. It is now generally accepted that plasma cells are the dominant cell type in the advanced lesion (71,72).

1.1.10 Recent advances in the understanding of pathogenesis

The role of host factors in determining an individual's susceptibility to periodontal disease has been elucidated by Shapira et al (73) in a fascinating case study. This reported the case of an otherwise healthy female who exhibited prepubertal periodontitis at age 10, juvenile periodontitis at 13, and rapidly progressive periodontitis at 29. Therefore, although these are considered distinct disease entities, which, as in this case, may manifest sequentially in the same individual, the pathology and pathogenesis of the disease appears to be similar.

The host response to oral micro-organisms and, in particular, to bacterial enzymes which are suspected as important virulence factors, appears to be relevant to the pathogenesis of periodontal disease. For example, argingipain, an arginine-specific cysteine protease of

Porphyromonas gingivalis, plays a key role as a virulence factor of this organism in the pathogenesis of periodontal disease via the direct destruction of periodontal tissue components and the disruption of normal host defence mechanisms (74). Targets for this enzyme include collagens I and IV and immunoglobulin G (IgG). Variations in host immune competence to eliminate or contain this organism are, therefore, crucial to perceived differences in subject susceptibility to periodontal disease. This issue will be discussed further in section 1.6.8.

The significance of various host factors in periodontal disease has recently been investigated. Platelet activating factor (PAF) which is a potent phospholipid mediator of inflammation has been shown to have saliva levels which correlate with the severity of periodontal inflammation (75). Prostaglandin E2 (PGE2) exerts a biphasic effect on IgG production in that high levels such as are found in inflammation attenuate the local IgG response, but reduced levels allow the local humoral immune response to recover (76). It was further reported that low dose PGE2 and interleukin-4 (IL-4) have a synergistic effect in raising local IgG production. The authors concluded that local PGE2 levels can regulate

immunoglobulin production and potentiate cytokine-induced class switching within gingival tissue. Takahashi et al (77) have studied the role of a number of cytokines in the induction of adhesion molecules on cultured human gingival fibroblasts, and have produced data supporting an interactive role for inflammatory cytokines and the expression of adhesion molecules on gingival fibroblasts in the pathogenesis of gingival inflammation in periodontal disease. The role of interleukins and adhesion molecules are discussed further in section 1.3.

1.1.11 Putative Immunological Mechanisms of Tissue Damage

Until fairly recently (78), discussions of the immunological mechanisms of tissue damage in periodontal diseases have referred to the four types of hypersensitivity reaction as classified by Coombs and Gell (79). However, the characteristics of these do not accord well with the clinical and histopathological signs.

Type I (anaphylactic), type II (cytotoxic) and type III (immune complex) reactions are all antibody-mediated

whereas type IV (delayed hypersensitivity) is cell-mediated. However, since B-cells and plasma cells predominate in the later stages of periodontitis, this cannot represent a pure type IV reaction.

Type I hypersensitivity reactions rely on IgE antibody bound to the surface of mast cells. Upon reaction with specific antigen, these cells release histamine and other inflammatory mediators. Mast cells have been demonstrated in the gingiva (80,81), as has IgE (82), but type I reactions are probably not important elements in the immunopathogenesis of periodontal disease, as reflected in the scarcity of mast cells and IgE antibody in the periodontal tissues.

Type II reactions rely on complement-fixing IgG and IgM antibody. Damaged and lysing cells have been demonstrated in periodontitis (83,84). This type of reaction is probably essentially protective. However, a recent study by Reinhardt *et al* (85) has shown a predominance of total IgG1 and IgG4 in active as opposed to stable periodontitis sites. Since IgG4 is relatively inefficient in fixing complement, two mechanisms suggest themselves here: 1) IgG1 is basically protective and a preponderance of IgG4 may promote disease progression by

failure of antibody/complement mediated bacterial destruction; and 2) a shift to IgG4 production locally may constitute an attempt to limit the potentially damaging effects of complement-mediated activity. Care should be taken in this interpretation, however, since immunoglobulin synthesised by gingival plasma cells has been shown to have specificity for non-oral bacteria (86) or non-bacterial antigens (87).

Type III reactions involve complement fixation by antibody-antigen complexes. However, since studies of complement in periodontal disease have demonstrated that these components are easily washed out, suggesting soluble proteins rather than fixed immune complexes occur (88), and attempts to extract insoluble immune complexes from periodontal tissue have not been successful (89), this type of reaction is unlikely to be important in periodontal destruction.

Another possible mechanism in the immunopathogenesis of periodontal disease is that of autoimmunity. Serum antibodies against type I collagen have been found in higher levels in periodontitis than in control subjects (90). A model has been presented whereby polyclonal activators initiate clonal expansion of autoreactive B-

cells, followed by a specific response to stimulation by autoantigens produced by the initial response (91). However, there is a general dearth of evidence implicating autoimmune mechanisms in the pathogenesis of periodontal disease.

1.1.12 Neutrophil dysfunction in the pathogenesis of periodontal disease

When an inflammatory process is initiated, the polymorphonuclear neutrophil (PMN) adheres to vascular endothelium via complementary receptors on the PMN and endothelial cell. Secondly, the PMN moves towards the cause of inflammation, e.g. invading bacteria, by chemotaxis i.e. directed movement along a concentration gradient generated by a chemoattractant. The opsonisation of the invading organism with immunoglobulin and complement components (e.g. IgG and C3b) stimulates phagocytosis (engulfment) by the PMN. The final stage of this process is microbial killing by discharge of the PMN granule contents either intracellularly (primary granules) or extracellularly (secondary and secretory granules).

The above processes are described in more detail in section 1.3.

Although the enzymes involved in oxidative killing are not released extracellularly, activated PMNs can produce superoxide anions on their surface which are then converted to hydrogen peroxide by peroxide dismutase. Combination of this with PMN myeloperoxidase and halide ions results in the formation of hypochlorous acid which is a potent oxidant capable of lysing gingival epithelial cells (92) and damaging periodontal ligament fibroblasts.

The presence of PMNs in the crevicular epithelium may protect the periodontal tissues against microbial attack by phagocytosing and ultimately killing the bacteria which can release lysosomal enzymes into the crevicular microenvironment (93). It is suggested that the neutrophils form a leucocyte wall interposed between the periodontal plaque mass and the junctional and crevicular epithelium. The leucocyte wall forms a digestive and secretory organ capable of protecting the delicate crevicular lining against bacterial injury (94).

Some studies have connected neutrophil dysfunction, e.g. defective chemotaxis, with the pathogenesis of early-onset forms of periodontitis (95-97). However, other

studies have found these parameters to be normal (98-100). Van Dyke reported in his review of 1991 (101) that local juvenile periodontitis (LJP) manifests with depressed neutrophil chemotaxis, bactericidal activity and leukotriene B_4 production. Phagocytosis of *Staphylococcus aureus* was also depressed, but, strangely, that of *Actinobacillus actinomycetemcomitans*, an organism associated with LJP, was not.

Therefore, it would appear that there is as yet no definitive evidence, with the exception of documented genetic disorders discussed in 1.1.13., linking neutrophil dysfunction with periodontal disease, although the normal functioning of these cells may, under certain circumstances, contribute to tissue damage.

1.1.13 Genetic factors in periodontal disease susceptibility

A significant association has been shown between a number of disorders with a genetic basis and periodontitis, e.g cyclic neutropenia (102), chronic idiopathic neutropenia (103), Papillon-Lefevre syndrome (104), trisomy 21 (105) and Chediak-Higashi syndrome (106).

Cyclic neutropenia is an autosomal dominant trait thought to be caused by a haemopoietic progenitor cell defect leading to episodic fluctuations in neutrophil numbers. The occurrence of severe generalised prepubertal periodontitis with rapid alveolar bone loss (107) highlights the importance of neutrophils as a major cellular defence against bacterial infections. Intrinsic abnormalities in lysosomal granule formation and mobilisation have been associated with defective intracellular bactericidal activity in neutrophils from patients with Chediak-Higashi syndrome (108). These neutrophil defects may predispose patients with this syndrome to severe periodontitis (109,110).

There are other genetically-linked disorders such as Ehlers-Danlos syndromes (111) which involve defects of collagen synthesis and cross-linking, or Papillon-Lefevre syndrome which seems to be caused by aberrant epithelial

development (112). All of the above disorders, however, involve systemic as well as periodontal manifestations.

In early-onset forms of periodontitis (EOP), however, only local disease occurs. Segregation analysis is a method of studying family trees (pedigrees) to formulate hypotheses concerning the mode of inheritance of a trait. By this method it has been established that there is a significant genetic component to some forms of EOP displaying autosomal dominant transmission (113). However, this is still problematic since other modes of inheritance cannot be excluded given the probable heterogeneity of EOP, and the difficulty of adducing mechanisms explaining the purely local disease manifestations.

Although there is no clear evidence linking adult periodontitis to genetic factors, there are data suggesting fundamental differences in individual responses to the same bacterial stimulus reflecting underlying genetic differences. For example, Garrison and Nichols (114) demonstrated that endotoxin-stimulated monocytes from periodontal disease-susceptible patients secreted more PGE2 than did endotoxin-stimulated monocytes from periodontal disease-resistant patients. Offenbacher et al

(115) describe a PGE2 host response model as an alternative paradigm of periodontal disease that places the emphasis on host response rather than bacterial aetiology. This is not to say that bacterial virulence properties are not important, but that host response may be the key factor in susceptibility.

1.1.14 Role of Antibody-mediated Mechanisms

The role of antibody-mediated mechanisms will be discussed at greater length in a later section. At this stage, it seems reasonable to state that the role of antibody in periodontal disease is primarily protective, but that deficiencies or functional failure of local and/or systemic antibody may lead to subject and/or site susceptibility. The pathogenesis of periodontal destruction is probably determined to a large extent by the pro-inflammatory and tissue-degrading effects of cytokines released in response to bacterial products. Much evidence has been adduced demonstrating the ability of oral bacteria to directly stimulate cytokine production by macrophages (116-119). LPS from oral Gram-negative

bacteria can markedly augment release of PGE_2 from cells activated by IFN-α (120), and polyclonal activators when present in optimal proportion to antigen can considerably amplify the secondary response to antigen (121). When one comes to consider the effects of these cytokines on metalloproteinase synthesis, prostaglandin release and bone resorption for example, the potential for pro-inflammatory and tissue-destructive events mediated by cytokines becomes obvious. The humoral immune response, insofar as it can bring about a reduction in bacterial antigen load, can be seen as acting against the promotion of tissue-destructive events.

1.1.15 Host Responses in Periodontal Diseases

It was demonstrated long ago that the gingiva adjacent to periodontal lesions is heavily infiltrated with mononuclear leucocytes. Models used in the earlier years of these studies suggested that the initial lesion is composed mainly of T-lymphocytes, with B-cells and plasma cells predominating at a later stage (34,122,123). However, this concept has recently been contested

(124,125). These studies found that both T-cells and B-cells may predominate in lesions, but that there was considerable variability in proportions of plasma cells. T4/T8 cell ratios are decreased in periodontal lesions compared with those seen in peripheral blood of periodontitis patients or in healthy gingiva (126,127). A recent study has demonstrated that many of the CD4+ cells in periodontal lesions are in fact memory cells, and that CD8+ cells suppress and macrophages promote immunoglobulin production by plasma cells in the gingiva (128). By these mechanisms, regulation of local immune responses by gingival cells is made possible.

Responsiveness of leucocytes to various stimuli has also been extensively studied in periodontal disease. Polyclonal B-cell activation, which involves hyper-reactivity of B-cells to bacterial products as indicated by their blastogenesis leading to massive production of lymphokines and immunoglobulin, has been shown to be increased in most periodontitis patients (91, 129). However, since this phenomenon cannot be demonstrated in about 30% of periodontitis patients, it may be that polyclonal B-cell activation offers another criterion by which these patients can be dichotomised into more

susceptible and less susceptible groups. This concept will be dealt with further in the discussion of antibody avidity.

Elevated monocyte response to LPS has also been demonstrated (114,130). These workers showed that periodontitis patients exhibit hyper-reactivity to LPS, presenting as increased monocyte PGE_2 and IL-1ß production, and suggested that these patients may be susceptible to disease progression because of this state of hyper-reactivity.

Degradation of soft tissue in periodontal disease can be effected by both bacterial and host enzymes, including the matrix metalloproteinases. These include collagenase which can be produced by both fibroblasts and PMNs. The fibroblast type degrades collagen type III, whereas the PMN type degrades collagen type I. The stromelysins have multiple specificity and can also superactivate the collagenases. Gelatinase can degrade collagen types IV and V and also breaks down denatured collagen. Birkedal-Hansen *et al* have shown that at least three suspected periodontopathogens, i.e. *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum*, produce factors that stimulate epithelial

cells to degrade collagen fibrils (131). The most likely mediator, in the case of *P. gingivalis*, is a thiol-dependent proteinase which activates mammalian procollagenase. In addition, the cytokines, IL-1ß, TGF-α and EGF, can stimulate keratinocytes to produce type I and type IV/V specific collagenase (132). The potent ability of certain bacteria to precipitate tissue degradation, not only indirectly via cytokines, but also directly, further emphasises the crucial role of the host response in eliminating or limiting these pathogens.

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

1.2 Microbiology of Periodontal Disease

1.2.1 Historical Perspective

Although references to periodontal diseases can be traced back to Roman physicians, oral microbiology can be said to date back to van Leeuwenhoek in 1772 (133). Later W.D. Miller, whose work was reprinted in 1973 (134), proposed

his acidogenic theory of dental caries and, in addition, proposed that "pyorrhoea alveolaris" is not caused by a specific bacterium occurring in every case (as in classical single-pathogen infections e.g. tuberculosis), but that various bacteria may be responsible.

As such this was one of the earliest expressions of what is now known as the non-specific plaque hypothesis. This postulates that dental plaque can be considered a homogeneous mass which causes periodontal disease once it has accreted to a point where it can overwhelm host defence mechanisms. Similarly, one of the first suggestions of the specific plaque hypothesis was that of Bass who suggested a specific micro-organism, "Endoameba buccalis" as the cause of periodontal disease, even going so far as to suggest a vaccine to this organism as a form of therapy (135).

1.2.2 Infectious Nature of Periodontal Disease

Since the periodontal diseases, comprising gingivitis and periodontitis, are caused by bacterial plaque, they are, therefore infectious diseases as borne out by much evidence summarised by Socransky (136). Notably, the

classic experimental gingivitis study of Loe *et al* (137) demonstrated a correlation between the build up of plaque and the development of clinically demonstrable gingivitis.

Secondly, periodontal treatment which reduces plaque mass and removes some key species altogether can be correlated with clinical improvement.

Thirdly, *in vivo* and *in vitro* studies of plaque micro-organisms provide further evidence of the infectious nature of periodontal disease, especially with regard to the specific plaque hypothesis. A number of case reports have demonstrated various infections caused by oral micro-organisms in extra-oral sites (138,139). Oral implantation into animals has been shown to cause periodontal disease with similar histopathology to that seen in humans (140,141). Mixed infections have also been shown to have synergistic effects (142).

In vitro studies of pathogenicity have investigated the ability of plaque bacteria to produce virulence factors. Since Gram-negative bacteria tend to predominate in periodontal pockets, high concentrations of lipopolysaccharide (LPS) endotoxin tend to build up. This causes a cytotoxic effect on host cells, promotes

osteoclastic bone resorption and results in a localised Schwartzman reaction with tissue necrosis.

Additionally, toxic metabolic end products are released and there are indirect effects such as activation of the alternative complement pathway (143).

Certain bacteria, notably *Porphyromonas gingivalis*, also produce enzymes capable of destroying host proteins (144). *P. gingivalis* and *Actinobacillus actinomycetemcomitans* both produce collagenase which is capable of degrading host collagen. *P. gingivalis* also produces enzymes capable of degrading host immunoglobulins (145) and this organism, along with *Prevotella* ssp., has been shown to be able to coat itself with Fab fragments of degraded IgA1 thus evading host defences (146). Another example of this sort of strategy is the bacterial capsule which can inhibit phagocytosis by polymorphonuclear leucocytes (PMNs) and macrophages.

By the production of enzymes such as collagenase, hyaluronidase, gelatinase, aminopeptidase, proteinase etc., plaque bacteria can increase the permeability of the epithelium lining the gingival sulcus, destroy connective tissue and thereby cause proliferation of the junctional epithelium apically along the root surfaces.

1.2.3 Microbial risk factors

There have been a number of studies which have set out to relate the bacterial composition of subgingival plaque samples with periodontal disease status. In a cross-sectional study of over 600 rural-dwelling blacks and whites older than 65 years of age, Beck et al (147,148) found that increased levels of periodontal disease were associated with several risk factors including increased counts of *Porphyromonas gingivalis* and *Prevotella intermedia* in black subjects and *Porphyromonas gingivalis* in white subjects. Grossi et al (149) discovered that *Bacteroides forsythus* and *Porphyromonas gingivalis* were positively associated with attachment loss in 1426 subjects who were older than 24 years of age. These cross-sectional studies support the idea of an important role for *Prevotella intermedia*, *Bacteroides forsythus* and especially *Porphyromonas gingivalis* in destructive periodontal diseases.

The above studies used immunofluorescent antibody techniques. However, a longitudinal study using the colony lift procedure and DNA probes (150) has demonstrated a relationship between ongoing attachment loss and sites with high levels ($>10^5$) of *Prevotella intermedia* and *Porphyromonas gingivalis*. The relative risk for *Porphyromonas gingivalis* was 2.8, compared with 1.6 for *Prevotella intermedia*. When the differences between mean bacterial counts at active and inactive sites were compared, marked differences spanning an order of magnitude were seen for a number of species including *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* serotype b.

Now that further new technology is available e.g. polymerase chain reaction (PCR) and checkerboard blotting, further light can be shed on the relationship between site disease status and microbiology.

1.2.4 Transmission of periodontal pathogens

The general impression given by much of the literature on periodontal microbiology is that each individual has his or her unique, endogenous microflora. However, these

organisms must come from somewhere. Vertical, i.e. parent to offspring, and horizontal transmission, i.e. between other individuals, have both been demonstrated by molecular epidemiology techniques. These involve fingerprinting DNA from strains recovered from different individuals. *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* isolated from parents and children have been shown to be identical to one another, but different from other families (151-154). On the other hand, transmission within married couples has also been demonstrated (155,156). Transmission between other species and humans is also possible (157).

The most dramatic evidence of transmission of these pathogens was the epidemic of acute necrotising ulcerative gingivitis, caused by fusiform and spirochaete infection, which occurred between 1915 and 1930 (158,159).

The emerging perspective is that person to person transmission may occur readily leading to new infections and recurrent infections throughout life.

1.2.5 Specific Micro-organisms

The infectious nature of periodontal diseases having been clearly established, a number of studies have implicated specific micro-organisms in the aetiology of these diseases (160-164). Classically, a pathogen has been defined by Koch's postulates (165). Briefly, these three requirements demand that 1) the agent must be isolated from every case of the disease, 2) the agent must not be isolated from cases of other disease states or from non-pathogenic (healthy) states and 3) after isolation and repeated growth in pure culture the agent must induce disease in experimental animals. However, Koch was required to abandon the third requirement in 1884 when he failed to induce cholera in animals, and in 1890 the second criterion was relaxed when the possibility of the carrier state was recognised.

Recent workers in periodontal microbiology have modified and extended Koch's postulates (164). The relevant criteria can be discussed under five headings. First, the criterion of association requires that the suspected pathogen be detected more frequently and at higher levels in disease cases than in controls. This would mean that the organism should be present in higher

levels in actively progressing sites than in non-progressing sites, healthy sites or sites showing improvement. It might also be expected that a system of longitudinal monitoring would show an increase in the pathogen prior to or concomitant with clinically assessed disease progression.

Secondly, the criterion of elimination is based on the premise that periodontal treatment should influence not only the clinical course of the disease but also the associated micro-flora. A corollary of this view is that failure to eliminate or at least reduce the level of the pathogen would eventually lead to further progression at that site.

Thirdly, the animal pathogenicity criterion continues to be used in the elucidation of possible pathogens, despite concerns about the use of animal models. They have been shown to produce valuable supportive evidence concerning the roles of certain micro-organisms in periodontal disease (166-168).

Fourthly, the response of the host to potential pathogens has been applied in attempts to differentiate the importance of various species in different forms of

periodontal disease. This will be discussed in greater detail at a later stage.

Finally, some suspected organisms have the capability to produce virulence factors and these may not only be important in disease initiation and progression, but may also be useful as indicators of the pathogenic potential of the organism (169).

1.2.6 Two prime candidates

Two organisms that have been particularly implicated in periodontal disease using these criteria are *P. gingivalis* and *A. actinomycetemcomitans*. Both of these organisms have been found in elevated numbers in periodontitis sites. However, *A. actinomycetemcomitans* has been associated particularly with the early-onset forms of periodontitis, localised juvenile periodontitis (LJP) and rapidly progressive periodontitis (RPP). It has also been detected in prospective studies of disease progression. Further evidence of association is that *P. gingivalis* has been shown to bind to epithelial cells (164).

Elimination of both organisms results in successful therapy and recurrent lesions were found to harbour these species. Both species have been shown to induce periodontal disease in animal studies (164). The host response in terms of specific antibody production, both local and systemic, has been shown to be elevated with both organisms in different types of periodontal disease, a high titre to *A. actinomycetemcomitans* being associated with LJP. Both organisms also produce a number of virulence factors including enzymes such as collagenase, numerous proteases in the case of *P. gingivalis* capable of destroying most host proteins, endotoxin, leucotoxin and epitheliotoxin in the case of *A. actinomycetemcomitans*, and also factors inducing bone resorption and inhibiting PMN function (164).

1.2.7 Other possible suspects

A number of other micro-organisms have also been implicated in periodontal disease. However, of these only spirochaetes, *Prevotella intermedia* and *Fusobacterium nucleatum* have been suggested as satisfying all of the

extended postulates (164). Spirochaetes are found in "superficial layers" of plaque near host tissues, as evidenced by electron microscopy, where they are actively motile, suggesting that they have a strong affinity for host tissue. Indeed, they have been shown to invade gingiva in acute ulcerative gingivitis (AUG) (170). However, since spirochaetes do not form colonies on agar media but instead form "spirochaetal haze" as they move through the agar when cultured by membrane-filter and agar-well techniques, and are also sensitive to mechanical forces during sample dispersion or dilution and particularly to atmospheric oxygen, the laboratory culture of spirochaetes has been fraught with problems.

F. nucleatum is a Gram-negative obligate anaerobe which is frequently isolated from subgingival dental plaque in patients with adult periodontitis. Indeed, high numbers are often detected in sites undergoing active periodontal destruction. However, high numbers of *F.nucleatum* can also be isolated in inactive sites and, therefore, the role of this organism in periodontitis is equivocal (164).

Prevotella intermedia, like *P. gingivalis*, is frequently isolated from periodontitis sites. A recent

study has demonstrated *Prevotella intermedia* in 88% of periodontitis subjects and *P. gingivalis* in 94% of these subjects. However, *Prevotella intermedia* was also detected in 42% of gingivitis subjects (i.e. no probing attachment loss > 2mm over one year of observation), whereas *P. gingivalis* was not detected in any of these subjects (171). Therefore, the association of *Prevotella intermedia* with periodontitis as opposed to gingivitis is also equivocal.

Since *P. gingivalis* and *A. actinomycetemcomitans* appear to be most strongly and unambiguously associated with periodontitis (172-175), the immune responses to these two organisms in particular were chosen for intensive study.

1.2.8 Specific antibiotic therapies for periodontal infections

Recently, some studies have attempted to relate specific antibiotic therapies to the presence of specific subgingival microflora. Slots et al (176) found that they had a small group of patients who had high counts of gram-

negative enteric rods in their subgingival plaque. These were successfully treated with systemic ciprofloxacin, which is effective against enteric bacteria. van Winkelhoff et al (177) suggested that amoxicillin plus metronidazole may be useful in controlling periodontal infections when *A. actinomycetemcomitans* is a major constituent of the subgingival plaque. Tetracycline was found by Haffajee et al (178) to be a useful adjunctive therapy when combinations of *P. gingivalis, Prevotella intermedia* and *B. forsythus* were predominant. However, augmentin seemed to be a better choice when these species were present in low numbers.

These examples illustrate the need for diagnostic tests which elucidate the nature of the microbiota in order to target antimicrobial therapy for periodontal infections more effectively.

1.2.9 Current Concepts of Aetiology

In 1992 Socransky and Haffajee summarised current concepts of the aetiology of destructive periodontal disease in their review (179). Pathogenic species have been discussed above, and host susceptibility will be dealt

with in the next section. However, the effect of beneficial species in the disease process is less clear, but it would seem reasonable to assume that non-pathogenic species could have a role in preventing disease initiation and progression by displacing pathogenic species.

Socransky and Haffajee conclude that the presence of a pathogenic species is necessary but not sufficient for disease to occur. In order that disease results from this pathogen 1) it must be of a virulent clonal type; 2) it must possess the chromosomal and extra-chromosomal genetic factors to initiate disease; 3) the host must be susceptible to this pathogen; 4) the pathogen must be in numbers sufficient to exceed the threshold for that host; 5) it must be at the right site; 6) other bacterial species must foster, or at least not inhibit, the process and 7) the local environment must be one which is conducive to the expression of the species' virulence properties.

It would seem to the cynical observer that the use of such extended requirements is an attempt to draw up a list of postulates which accord with what is already known about suspected periodontopathogens. However, their value can only be judged in truly prospective studies. In

addition, the demonstration that virulence factors can be transmitted between different strains and even between different species, as evidenced by the transfer of antibiotic resistance between *P. denticola* and *P. intermedia* (180), may have enormous implications. The importance of bacteria in periodontal aetiology may in future be ascribed to genes coding for specific virulence factors rather than to particular micro-organisms.

Socransky and Haffajee (179) conclude that diagnostic tests for bacteria should probably not exist in isolation but in combination with other diagnostic tests of host susceptibility. It is to this latter aspect in terms of humoral immune response that this study is applied.

1.3 Some aspects of basic immunology relevant to periodontal disease

1.3.1 Introduction

Immunology has advanced at a rapid pace in recent years and many of these developments have impacted significantly on research into chronic immune and inflammatory diseases such as periodontal disease. These developments have been synthesised in a number of recent textbooks (181-185) and only a summary of the relevant points is presented here.

1.3.2 Innate immunity

Immunity to infection falls into two main categories, innate or non-specific immunity and acquired or specific immunity.

Mechanisms of innate immunity operate regardless of any prior contact with the invading organism. The simplest and most basic of these mechanisms are physical barriers preventing access to the body. These include skin which is impermeable, when intact, to most micro-organisms. In addition, mucus is secreted by membranes lining the inner surfaces of the body and this not only

prevents adherence of micro-organisms to epithelial cells but also traps them allowing them to be eliminated mechanically by e.g. movement of cilia within the lungs.

The washing action of fluids such as tears, saliva and urine also help keep epithelial surfaces clear of invading organisms and many of the secreted body fluids contain bactericidal agents e.g. lysozyme in tears, saliva and nasal mucus, lactoferrin and lactoperoxidase in milk, and hydrochloric acid secreted by the stomach. A similar beneficial washing function might be attributed to gingival crevicular fluid (GCF) also. This will be discussed at a later stage.

The normal bacterial flora of the body can also act as an effective buffer against infection by both passive and active mechanisms. They can inhibit the growth of pathogenic organisms by competition for nutrients or production of inhibitors. Commensal organisms in the gut can produce colicins. These bactericidins bind to the negatively-charged surface of susceptible bacteria and insert a hydrophobic, helical molecule into the membrane. This molecule then becomes completely hydrophobic and forms a voltage-dependent channel in the

membrane, destroying the cell's electrical energy potential and leading to cell death.

1.3.3 Molecular Factors

If this first cohort of defence strategies is circumvented, the two main remaining innate mechanisms are those dependent on molecular factors and those dependent on cells.

Lysozyme is probably the most ubiquitous and abundant of secreted anti-bacterials, a muraminidase which can split the peptidoglycan cell wall of susceptible bacteria.

Endotoxins produced by micro-organisms during infection stimulate the production of the cytokines, interleukin-1 (IL-1) and IL-6. IL-1 is an endogenous pyrogen (a general increase in body temperature being another innate defence mechanism), and can also, along with IL-6, stimulate the liver and other organs to produce molecules such as α1-antitrypsin (α1-AT), α2-macroglobulin (α2-M), both potent inhibitors of human proteases, and C-reactive protein (CRP); proteins produced in this way as a response to infection are known as acute-phase proteins.

CRP binds in a Ca-dependent way to micro-organisms which contain membrane phosphorylcholine. Once complexed,

the CRP-bound particle activates complement through the classical pathway resulting in binding of complement C3b to the membrane. This process, called opsonisation (literally "making ready for the table"), renders the cell susceptible to adherence of phagocytes. Both phagocytic cells and complement will be discussed later. Interestingly, molecules such as CRP appear to be highly conserved in evolutionary terms, since the horseshoe crab's haemolymph contains a very similar analogue, limulin.

Finally, interferons are a group of broad-spectrum anti-viral agents. They are so named because they were first recognised by the phenomenon of viral interference. This is the mechanism by which an animal infected with one virus is rendered capable of resisting superinfection by another virus.

Cells produce interferon when infected by a virus and secrete it into the extracellular fluid. The interferon then binds to uninfected cells. This promotes derepression of two genes in the target cell, permitting the synthesis of two enzymes. One of these is a protein kinase which catalyses the phosphorylation of a ribosomal protein and an initiation factor necessary for protein

synthesis, thereby preventing mRNA translation to a large extent. The other catalyses the formation of a short adenylic acid polymer which activates a latent endonuclease. The latter has the ability to degrade both viral and host mRNA. The overall effect of this mechanism is to create a "cordon sanitaire" of uninfected and uninfectable cells around the locus of infection, thus ensuring containment.

However, as this mechanism can also inhibit host cell division as effectively as viral replication, it could have implications also in immune surveillance of neoplastic cells. By the same token, interferons could conceivably have a role in the healing phase of periodontal disease by "damping down" the immune and inflammatory reactions to bacterial products once the challenge has been removed.

1.3.4 Cellular factors

Interferons may also modulate the activity of natural killer (NK) cells, which brings us to a discussion of the cellular components of innate immunity.

NK cells are large granular lymphocytes which have membrane receptors thought to be capable of recognising structures on high molecular weight glycoproteins expressed on the surface of virally infected cells. Via these receptors, the NK cell is brought close to the target cell and becomes activated leading to polarisation of granules between nucleus and target, and extracellular release of their contents into the intercellular space. Among the proteins released is perforin or cytolysin which bears some structural homology to complement C9. It can bind to the target cell membrane and form a transmembrane pore effecting apoptosis (programmed cell death) via a Ca-dependent endonuclease.

Another cell which effects extracellular killing is the eosinophil, a polymorphonuclear leucocyte which can bind to large parasites, e.g. helminths, coated with C3b. Most of these parasites are resistant to C9. The eosinophil granules contain a cationic protein and a major basic protein, both capable of damaging the parasite membrane, and also a protein capable of forming a transmembrane pore similarly to C9 or perforin.

The two main cells involved in killing of micro-organisms by phagocytosis, a form of total encirclement by

the cell followed by intracellular destruction and digestion, are macrophages and neutrophils, the so-called "professional phagocytes".

Promonocytes in the bone marrow differentiate into mature monocytes which disseminate through the bloodstream to locate in a number of tissues as mature macrophages. These macrophage populations make up the mononuclear phagocyte system, which comprises alveolar macrophages in the lung, cells in the lining of spleen sinusoids and lymph node medullary sinuses, Kupffer cells in the liver, and also macrophages throughout the connective tissue and around the basement membrane of capillaries. They also exist as osteoclasts in bone, mesangial cells in the kidney glomerulus and microglia in the brain. Macrophages are long-lived cells which can phagocytose invading bacteria directly. However, their particular specialism is their ability to phagocytose host cells infected with bacteria, protozoa or viruses.

The polymorphonuclear neutrophil (PMN) is a polymorphonuclear cell similar to the eosinophil but considerably more abundant, constituting about 70% of the leucocytes in the peripheral blood. These are short-lived cells produced in vast numbers in the bone marrow and

spending about 36 hours in the bloodstream upon maturation before migrating into the tissues. PMNs contain three types of granules. The primary azurophilic granules contain myeloperoxidase, a group of cationic proteins and also some lysozyme. The secondary, so-called "specific", granules contain lactoferrin and large amounts of lysozyme. Acid hydrolases are contained in the tertiary granules. Since PMNs contain large glycogen stores which can be metabolised by glycolysis, these cells are ideally suited to operate under anaerobic conditions; a factor of crucial importance in periodontal disease.

However, these cells do not operate in isolation. For example, activation and emigration of PMNs into a site of infection, e.g. a periodontal pocket, involves interaction between PMNs, macrophages and the complement system.

1.3.5 Complement

Complement comprises a complex series of 20 plasma proteins which constitute a triggered enzyme system similar in mechanism to the blood clotting, fibrinolytic

and kinin-forming systems. Like these, it operates as a cascade where the product of one reaction then acts as an enzyme in the next. The net effect of this is to produce a rapid and highly amplified response to an initial trigger stimulus.

1.3.6 Regulatory Cell Surface Receptors

As previously discussed, the interactions between immune cells are not only influenced and controlled by soluble agents, e.g. cytokines, complement and immunoglobulins, but also by regulatory cell surface molecules. These fall into four categories on immune cells: 1) MHC receptors; 2) cell surface receptors; 3) cluster of differentiation (CD) antigens; and 4) adhesins.

MHC receptors are transmembrane heterodimers. MHC class I molecules consist of a 43kDa peptide heavy chain non-covalently linked to a smaller 11kDa peptide called β-microglobulin. Most of the heavy chain is organised into three globular domains which protrude from the cell surface. A hydrophobic domain anchors the molecule in the membrane and a short hydrophilic sequence carries the C-terminus into the cytoplasm. Class II MHC receptors are

also transmembrane glycoproteins, consisting of an α-chain of 34kDa and a β-chain of 28kDa. Class I receptors are found on virtually all cells, whereas class II receptors are found in B-cells and macrophages, and can be induced by γ-interferon on capillary endothelial and epithelial cells. In addition, a number of other genes within the MHC chromosome 6 region are known as MHC class III. These include genes coding for complement factors C2, C4 and factor B, and also TNF-α and -ß. The MHC system has evolved as a highly polymorphic system based on multiple alleles (i.e. alternative genes at each locus). These multiple allelic forms are produced by various means including recombination, homologous but unequal cross-over, point mutation and gene conversion. This latter mechanism involves transfer of short nucleotide sequences from apparently non-functional genes, e.g Qa, into functional ones.

The importance of MHC receptors in immunological phenomena has already been discussed, but they also have influence in developmental and hormonal events.

The T-cell receptor (TCR) merits further discussion. Like the MHC receptor it is also a heterodimer, comprising

two chains of 40-50kDa each. Unlike B-cell receptors which exist as membrane-bound immunoglobulin monomer, the TCR is obviously not synthesised by immunoglobulin genes. Functional T-cells display the TCR on their surface non-covalently bound to CD3 (one of the cluster of differentiation antigens). The seven-peptide chain CD3 transduces the antigen recognition signal into the cell.

Although the α and ß chains of the TCR are not related to immunoglobulin, they share the same function. Each chain folds into two domains, one having a relatively invariant structure, the other displaying great variability akin to that of the immunoglobulin Fab fragment.

The CD antigens were discovered by immunising animals with human lymphocytes. The antibodies produced recognise particular lymphocyte sub-populations. For example, CD3 is present on all T-cells but not on B-cells. CD4 is expressed on T-helper cells and CD8 on cytotoxic T-cells. These latter CD antigens act as co-receptors for class II MHC-antigen complex and class I MHC-antigen complex respectively, and are part of the TCR complex. CD4 is also a receptor for the human immunodeficiency virus.

The adhesins fall into four main groups: 1) integrins; 2) adhesion molecules of the immunoglobulin supergene family; 3) lectin-like adhesion molecules, the selectins; and 4) the CD44, or Hermes group, which interact with ligands called addressins on vascular endothelium.

Important integrins include CR3 and CR4 which bind to complement C3b and LFA which binds to ICAM-1 and ICAM-2. ICAM-1 and ICAM-2 are both found on capillary endothelium, and ICAM-1 is also expressed on B-cells and T-cells, and is probably involved in the interaction between antigen-presenting cells and lymphocytes. ICAM-1 is also probably important in transendothelial migration of PMNs.

Selectins include endothelial/leucocyte adhesion molecule (ELAM). Binding of neutrophils to ELAM is thought to be a crucial first step in neutrophil emigration across the capillary endothelium.

The CD44 homing receptors expressed on lymphocytes appear to be important in the binding of these cells to the high endothelial venules (HEV) of peripheral lymph nodes. The ligands for the CD44 antigen found on lymphocytes have been called vascular addressins. It is thought that there are other homing receptors selective

for other lymphoid tissues, e.g. Peyer's Patch HEV, and, therefore, these receptors may be crucial in communicating lymphocytes to specific sites.

1.3.7 Initiation, maintenance and resolution of inflammation

The original inflammatory stimulus typically results in the release of thrombin or histamine which upregulate the surface expression of P-selectin and platelet activating factor (PAF) on the endothelial cells lining the venules. The polymorphonuclear neutrophil (PMN) surface mucins contain sialyl Lewis x carbohydrate ligands which engage with the lectin-like domain at the terminus of P-selectin. This causes the neutrophil to roll along the endothelial wall and helps PAF to bind with its corresponding PAF-R receptor on the PMN, increasing the efficiency of PMN activation.

This makes the PMN more susceptible to chemotactic agents and, once adhesion has been stabilised by binding of PMN LFA-1 to endothelial ICAM-1, C5a and leukotriene B-4, stimulate the exit of the PMN from the circulation by movement across the basement membrane between endothelial

cells and along the chemotactic gradient to the inflammation site. This is known as diapedesis.

Other stimuli such as damage to the basement membrane of the vascular endothelium and bacterially-derived toxins e.g. lipopolysaccharide (LPS) cause activation of endothelium and increased vascular permeability via plasmin, thrombin, bradykinin and C3a. Thus bacterial toxins act at a later, ongoing or maintenance phase, of the inflammatory process by stimulating macrophages to produce interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α). Clearly this bacterial ability to maintain inflammation if not removed by immunological or other processes is of particular relevance to the micro-environment of the periodontal pocket.

Lipopolysaccharides (LPS) and carbohydrates produced by bacteria react with receptors on the membranes of macrophages, e.g. the mannose/fucose receptor and the complement receptor. The mannose/fucose receptor binds surface carbohydrates of the bacterium whereas the CR3 complement receptor binds bacteria which have been opsonised with C3b via the alternative pathway. Macrophages so stimulated release IL-1 and tumour necrosis

factor (TNF), and synthesise and release neutrophil chemotactic factor (NCF; also designated IL-8).

These factors diffuse into the local vasculature and stimulate vascular endothelial cells to express the endothelial leucocyte adhesion molecule, ELAM-1, and up-regulate the concentrations of the adhesion molecules, ICAM-1 and ICAM-2. PMNs in the adjacent circulation are then bound by these adhesion molecules via their own adhesin, LFA-1. Movement of the PMN through the vascular endothelium and the basement membrane is modulated by IL-1 and TNF, this action being independent of any existing chemotactic gradient. However, the neutrophil's further journey through the subendothelial extracellular matrix occurs by chemotaxis through a chemotactic gradient, a form of directed migration. Chemotaxins involved here include IL-8, C5a, bacterially-derived formyl peptides, e.g. formyl-methionyl-leucyl-phenylalanine, and the leukotriene, B4.

These chemotaxins also activate the PMNs which can then destroy opsonised bacteria (i.e. with bound antibody/complement or complement only) by a number of oxygen-dependent mechanisms involving lipid peroxidation and the peroxide-myeloperoxidase system. As previously

mentioned, PMNs can also kill using oxygen-independent mechanisms, e.g. neutral serine proteases (proteinases, elastase, cathepsin G), lactoferrin, lysozyme and defensins. These latter mechanisms are probably of much greater relative importance in the anaerobic micro-environment of the periodontal pocket.

Normally, inflammation can be resolved. This involves:-

1) acute-phase proteins acting as protease inhibitors;

2) agents active at the cellular level e.g. a) prostaglandin E2 inhibits cytokine production by T-cells and macrophages, b) T-cell growth factor ß (TGF-ß) deactivates macrophages by inhibiting the production of reactive oxygen intermediates and downregulating major histocompatibility complex class II expression and natural killer (NK) cell cytotoxicity, c) IL-10 inhibits antigen presentation, cytokine production and nitric oxide (NO) killing by macrophages, d) binding to the surface proteins on monocytes and neutrophils by lipocortin-1 controlled by endogenous glucocorticoids produced via the hypothalamic-pituitary-adrenal axis.

When the inflammatory agent has been cleared from the tissue, the above regulatory processes will normalise the

site. If the inflammation has traumatised the tissue through its intensity and extent, TGF-ß plays a major role in the subsequent wound healing by stimulating fibroblast division and the laying down of new extracellular matrix elements via e.g. increased tissue inhibitor of metalloproteinases (TIMP) production.

However, chronic inflammation can develop if the cause of the inflammation, e.g. an invading organism, cannot be eliminated by the immune system. The site becomes dominated by macrophages, often activated. Some form arrays of epithelioid cells and others fuse to form giant cells. Lymphocytes also infiltrate and a granuloma is formed. The specifics of chronic inflammation in periodontal disease are dealt with in section 1.1.

1.3.8 Role of B-lymphocytes in acquired immunity

The cells mainly involved in specific or acquired immunity are antigen-presenting cells and lymphocytes. The former include macrophages, dendritic cells in the follicles of the lymph nodes, Langerhan's cells of the skin and mucous membranes, and B-lymphocytes themselves. These cells present antigen to B-lymphocytes and T-helper lymphocytes.

The activated T-helper cells then produce cytokines which stimulate B-cells to differentiate into antibody-producing plasma cells.

Early workers in the field of antibody production postulated that antibody was produced as a malleable, uniform molecule which could be moulded to a specific structure by using the antigen as a template. However, Ehrlich (186), even a century ago, anticipated the mechanism which is accepted today, that of clonal selection by antigen. B-cells differentiate in the bone-marrow and are programmed to produce only one antibody. They display this on their surface as a receptor (about 10^5 molecules) but produce no further antibody (i.e. they are quiescent). When these antibody receptors are bound by antigen a triggering signal causes the B-cell to develop into an antibody-producing plasma cell.

The second step in this process is that the activated B-cell proliferates to produce a clone i.e. a large number of identical cells producing identical antibody. This is known as clonal expansion and allows a high level of serum antibody to be produced within a few days.

A third feature of this process is that clonal expansion not only produces antibody-producing plasma

cells but also a proportion of quiescent cells expressing antibody on their surface. These memory cells provide a relatively large pool ready to produce a much more rapid and intense antibody response on further antigen challenge.

1.3.9 Role of T-lymphocytes in acquired immunity

T-cells act by another mechanism to destroy invading viruses, mycobacteria and protozoa which attempt to evade host defences by proliferating inside host cells. T-cells differentiate within the thymus and will only recognise foreign antigen when it is on the surface of a host cell in association with cell surface markers of the major histocompatibility complex (MHC). These regulatory cell surface molecules will be discussed in depth later.

Killing of virally-infected cells can be achieved by two mechanisms, one involving NK cells and the other cytotoxic T-cells. Apart from their direct effect on virally-infected cells, as already discussed, NK cells can also kill these cells by the process of antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells have a

receptor for the Fc portion of antibody and can thus bind to antibodies directed against virally-coded surface antigens. The ADCC mechanism works well *in vitro*, but its relevance *in vivo* is difficult to assess.

However, a subpopulation of cytotoxic T-cells produces a wide range of surface receptors similar to the surface antigen receptors on B-cells. Similarly, these T-cells are programmed to recognise only one antigen when it is found in association with a class I MHC receptor. The cytotoxic T-cell can thus bind to the infected cell and destroy it.

T-helper cells will bind to an antigen for which they are programmed when it is found in association with a class II MHC receptor on the surface of an infected macrophage. They then produce lymphokines, including γ-interferon and other macrophage activating factors, re-triggering the microbiocidal mechanisms of the macrophage which had been repressed by the intracellular pathogen. Cytotoxic T-cells are also capable of acting in this way by producing γ-interferon. These actions of T-lymphocytes are referred to as cell-mediated immunity. In a similar fashion to that which pertains for B-cells, T-cells are selected and activated by antigen and expanded by clonal

proliferation to produce a large clone of activated T-cells and also a pool of quiescent memory cells.

1.3.10 Immunoglobulins

An antibody, or immunoglobulin, molecule is made up of four peptide chains, two identical heavy chains and two identical light chains linked by interchain disulphide bonds. Immunoglobulin G (IgG) can be split by proteolytic enzymes to produce fragments giving clues to their function. The Fab fragment is now recognised as the antigen-binding fragment and the Fc as having other functions, e.g. complement binding. The Fab fragment contains considerable variability whereas the Fc fragment is relatively constant, and analysis of amino-acid variability has identified three hypervariable sequences on the heavy chain and three on the light chain in the Fab fragment.

Five different types of heavy chain exist in humans and these define the immunoglobulin classes, IgG, IgA, IgM, IgD and IgE. These are termed the immunoglobulin

classes. IgG is the principal immunoglobulin produced in the secondary immune response. IgG diffuses more easily than the other immunoglobulins into the extracellular spaces where it has the predominant role in neutralising bacterial toxins and binding to micro-organisms to promote complement fixation followed by phagocytosis.

The unique biological function of different immunoglobulin classes is mediated by the Fc portion. In the case of IgG, monocytes and macrophages contain receptors for Fcγ (FcγRI). FcγRII is found on monocytes, neutrophils, eosinophils, platelets and B-cells. IgG complexes can bind to platelets causing thrombosis. Stimulation of B-cell Fc receptor leads to downregulation of IgG production, a possible mechanism whereby IgG and IgG immune complexes exert a negative feedback effect on antibody production.

The Fcγ region of IgG is responsible for complement activation via the classical pathway, binding to C1q and causing its activation.

Another low-affinity Fc receptor, FcγRIII, which is found on NK cells, macrophages, PMNs and eosinophils, is

thought to be responsible for mediating ADCC by NK cells and immune complex clearance by macrophages.

IgG possesses the unique property among immunoglobulin classes of being able to cross the placenta providing considerable protection to the newborn in the first weeks of life, further supplemented by IgG in milk. The transport processes required here involve translocation of IgG across the cell barrier by complexing with another Fcγ receptor. A recent study has shown that the FcγRn receptor in the gut cells of the newborn rat complexes with IgG at relatively low pH in the gut lumen. This complex dissociates at the higher pH of the basal layer once the IgG has been transported across the intestinal cell (187). However, this mechanism has been only been demonstrated in rodents.

IgA is adapted to defend the exposed external and internal surfaces of the body against micro-organisms. It is found predominantly in sero-mucous secretions, e.g. lung secretions, saliva and secretions of the gastrointestinal and genito-urinary tracts. In these fluids it is present as a dimer which is stabilised against proteolysis by combination with the secretory component, which is synthesised by local epithelial cells.

Dimerisation occurs intracellularly via a joining sequence (J-chain), to prevent association of monomers with differing specificity. Essentially, IgA antibodies coat micro-organisms and thereby inhibit their adherence to mucosal cells. Aggregated IgA can also bind PMNs and can activate the alternative complement pathway. Monomeric IgA is also found in relatively high levels in the circulation indicating an additional systemic role for IgA.

IgM exists as a pentamer joined by a J-chain. IgM antibodies, although of low affinity as measured against single determinants, can demonstrate relatively high avidity, i.e. overall binding strength with molecules containing multiple epitopes. This is because the multivalency of IgM confers a synergistic effect whereby the binding of the molecule to two epitopes is of many times greater strength than the sum of the two interactions. In fact, the avidity is determined by a multiplication of the two binding strengths. This bonus effect of multivalency can be thought of as a vast reduction in the probability of simultaneous dissociation of several interactions as opposed to a single interaction.

IgM appears in the primary immune response and is extremely efficient in agglutination and cytolysis of invading cells. As such it is thought to play a vital role in control of bacteraemia.

Immunoglobulins are further grouped into subclasses. IgG, for example, exists as IgG1, IgG2, IgG3 and IgG4, the difference being in the heavy chains which exist as γ 1,2,3 and 4 respectively. These heavy chain differences give rise to differences in biological properties. IgG2, for example, is relatively inefficient in placental transfer, whereas IgG3 is a very avid binder of complement, and IgG4 is a poor fixer of complement. IgG1 and IgG3 both promote strong binding of monocytes. Some of these differences between different IgG subclasses may have an impact on the course of periodontal disease, and this will be discussed later.

The hypervariable regions on the immunoglobulin molecules which form the antigen-binding site are themselves unique antigens which can be recognised by antibodies raised against them. In this context, these regions are referred to as idiotypic determinants; these compare with the isotypic determinants which define the

various class and subclass variants and allotypes which define genetic variants of various regions.

Jerne's network hypothesis (188) states that because lymphocytes can recognise a vast array of foreign antigens, they should also be able to recognise the idiotypes of other lymphocytes. It is thought that a network may be formed which relies on idiotype-anti-idiotype recognition between lymphocytes of different subsets. The response to foreign antigen would then affect the equilibrium of this network and provoke an appropriate response.

Interestingly, it has been shown that if hybridomas are formed by fusing myeloma cells with spleen cells from foetal mice which are just beginning to secrete immunoglobulins, then an unusually high proportion are selected as idiotype-anti-idiotype pairs. This high level of idiotype connectivity involving CD5 B-cells is echoed in the so-called "natural antibodies" which appear spontaneously in germ-free animals not exposed to exogenous antigens. In both cases the antibodies produced have specificity for autoantigens such as heat-shock protein hsp65 and also highly-conserved microbial antigens

such as hsp65 which have several potential epitopes identified with the self homologue.

These findings have given rise to the concept of a CD5 B-cell population forming an inward-looking world in which the component cells recognise and stimulate one another constantly through their idiotypic receptor interactions to produce a range of IgM antibodies which provide an early general, or "broad-brush", defence against infection. Indeed Cohen and Young (189) have expounded the idea of an immunological homunculus in which a functional picture of the body is encoded within the immune system by regulatory committees of B- and T-cells which recognise certain dominant self-antigens representing the major organs of the body. This is analogous with the neurological homunculus which is a functional picture of the body mapped onto the cerebral cortex in which the space occupied by a given neural network is directly related to the neurological importance of the organ it encodes, e.g. human visual and speech organs and canine olfactory organs are prominently represented.

An illustration of how this mechanism might work would be CD5 B-cells bearing surface receptors for self-

hsp65 will, in an infection, selectively focus the bacterial hsp65 onto their surface receptors, making it dominant over other bacterial antigens, and process it. The self-epitopes will be recognised by autoreactive T-cells which are highly regulated within the homunculus; whereas T-cells specific for the non-self hsp65 epitopes are not so constrained and will generate an effective immune response.

This idiotype connectivity between self and highly-conserved bacterial antigens may thus be regarded as a means of downregulating self-reactivity whilst providing an enhanced general response to bacterial antigens upon initial exposure. However, in the context of susceptibility to periodontal disease, it is fascinating to speculate on foetal exposure to bacterial antigen, as has been postulated by Lopatin et al (190), especially if this involves non-conserved (i.e. species-specific) antigen exposure prior to commencement of immunoglobulin production. This will be discussed further in section 1.4.

1.3.11 Antigen-antibody binding

There are four main intermolecular forces involved in antigen-antibody interactions:-

1) Electrostatic forces caused by the attraction between ionic groups with opposite charge on the two molecules. Typically between proteins, this could be an amino group, NH_3^+ on e.g. lysine, and a carboxyl group, CO_2^- on e.g. glutamine. The force of attraction is expressed as $F \propto 1/k_D d^2$. Therefore, the attractive force increases by a factor of four as the separation is halved. Water has a very high dielectric constant (k_D), so the elimination of water molecules as the interacting groups are forced together further increases F. Moreover, electrostatic forces can be generated by electron transfer reactions between antigen and antibody.

2) Hydrogen bonding occurs via the innate polarity (not charge) of groups such as hydroxyl (OH) and amino (NH_2) causing mutual attraction. It may be mediated via water molecules. whose hydrogen bonding leads to the innate viscosity of water. However, since this is essentially an electrostatic interaction, the elimination of water

increases the binding energy by reduction in dielectric constant.

3) Similarly, hydrophobic bonding between antigen and antibody is mediated by hydrogen bonding between water molecules. Thermodynamically, a higher entropy, lower energy state is produced in the surrounding aqueous environment if hydrophobic groups on adjacent molecules come together to exclude water molecules. It has been estimated that hydrophobic forces may contribute up to 50% of the total strength of the antigen-antibody bond.

4) Van der Waals forces result from a temporary perturbation of electrons in one molecule effectively forming a dipole which induces a dipolar perturbation in the other molecule. The two dipoles then have a force of attraction between them generated by the dipolar oscillation as the displaced electrons swing back through the equilibrium point and beyond. The force produced here is expressed as $F \propto 1/d^7$ and depends on the interaction between the external electron clouds on atoms of adjacent molecules.

In general, the complementary electron cloud shapes on the combining site of the antibody and the surface determinant of the antigen enable the two molecules to fit

snugly together so that the intermolecular distance becomes very small and the nonspecific protein interaction forces (given above as 1-4) are considerably increased.

The affinity of a monovalent antigen-antibody bond can be expressed as a dissociation constant for the interaction. However, in the natural situation where an individual has been immunised with a multivalent antigen, a bonus effect occurs in which the dissociation constants are multiplied together to derive the overall dissociation constant known as functional affinity or avidity. This is because antibodies which bind more than one antigenic site (epitope) can only be dissociated by the simultaneous breaking of two or more bonds. The probability of two simultaneous events is equivalent to the product of the probabilities of the two independent events.

The role of antibody avidity in periodontal disease will be discussed further in section 1.6.

1.3.12 Generation of Diversity of Antibodies

Since the immune system must be capable of producing millions of different antibodies, the dedication of a

single gene to programme for each antibody is clearly not an option as the mechanism producing such diversity.

To illustrate how such diversity is generated from a limited gene pool, the genes coding for heavy chains will be discussed here. The first element is that there are several genes coding for these; a single cluster is formed by the subclass constant region genes, and there is also a group of twelve highly variable D segments inserted between the V segment coding for the hypervariable regions and the J segment coding for a joining sequence to the constant region.

The diversity latent in this pool is considerably amplified by various mechanisms. The first of these involves rearrangement of these genes as the precursor cell differentiates into a mature B-lymphocyte. For example, there are 500 different V segments, 15 different D segments and 4 different J segments coding for heavy chain hypervariable region. Possible VxDxJ combinations generate thirty thousand rearrangements.

A second mechanism involves splicing out of base triplets upon recombination of V, D and J genes resulting in removal of one amino-acid from the protein sequence. Splicing out of single base pairs and doublets can also

occur resulting in a frame shift which thereafter codes for a completely different protein sequence.

A third mechanism involves random association between heavy chains and light chains, both of which have hypervariable regions. Since possible light chain combinations are of the order of 10^3, total heavy/light chain combinations are on the order of 10^7-10^8.

Other mechanisms amplify this diversity yet further, e.g. V gene swapping and reading of D genes in three different reading frames.

A final mechanism which deserves specific attention is that of somatic mutation. The V region rate of somatic mutation as a result of single base substitutions has been assessed at 2-4%, with mutations being more prevalent in IgG and IgA than in IgM. It is thought that somatic mutation is more involved in class switching and affinity maturation in association with immunological memory generation than simply in the generation of greater antibody diversity.

These and other mechanisms which tune the humoral immune response to pathogens in terms of avidity, subclass etc. during the course of periodontal disease will be discussed in more depth later.

1.4 Other factors relevant to the aetiology of periodontal disease

1.4.1 Introduction

A number of other factors are worthy of consideration as potentially relevant to the development of periodontal disease. Here, smoking behaviour, ageing and the induction of oral tolerance are chosen for further discussion, both because of their intrinsic value in the elucidation of this disease process and for their relevance to the present study.

1.4.2 Smoking and periodontal disease

MacGregor has extensively reviewed the effects of smoking on plaque formation, the oral microflora, the host response and periodontal wound healing (191).

It has been established that there is no significant difference in the rate of plaque growth between smokers and non-smokers (192-194). However, a number of epidemiological studies have shown that smokers have poorer oral hygiene and thus greater accumulation of

plaque than non-smokers. Ainamo (195) conducted a study of 167 male individuals aged 18-26 and found mean plaque index (PI) (196) to be lower in non-smokers, increasing linearly with increasing cigarette consumption. An identical twin study by Bergstrom and Flolerus-Myrhed demonstrated higher levels of plaque in smokers than in non-smokers, and also higher levels with increasing smoking exposure (197). This is particularly significant because endogenous factors were taken into account by pairing genetically identical individuals.

It has been established that smokers and non-smokers differ in personality traits in that smokers tend to be more extrovert, exhibiting more developed grooming behaviour but, at the same time, less well developed health-related behaviour (198). This bears out previous studies showing that smokers spent less time toothbrushing and brushed less adequately than non-smokers (192,199). Therefore, the increased rates of plaque accumulation in smokers, which clearly have a bearing on the development of periodontal disease, seem to be attributable to differences in toothbrushing behaviour.

Oral bacteria have been shown to be susceptible to the effects of tobacco smoke *in vitro*, with some species

being differentially affected. *Neisseriae, Streptococci* and *Staphylococci* have been shown to be particularly vulnerable to the effects of smoke (200). On the other hand, tobacco smoke has a strong reducing effect in the mouth (201), which might promote the growth of anaerobic bacteria by the elimination of oxygen. This might explain the marked association between smoking and acute necrotising ulcerative gingivitis (ANUG) (202), which is caused by obligate anaerobic spirochaetes and fusiforms.

Smoking has been shown to cause vasoconstriction and a decrease in blood flow in the oral mucosa (203). This may confound the assessment of periodontal status when bleeding on probing is used as a parameter. Moreover, if host defences are reliant on systemic input of cells and serum components, the supply of these may be reduced in smokers.

Holt et al (204) found that cells of the immune system were highly susceptible *in vitro* to tobacco smoke exposure compared with epithelioid and fibroblastic cells. Noble and Penny (205) noted that circulating PMNs from habitual smokers had a reduced rate of chemotactic migration compared with non-smokers. Therefore, immune and inflammatory reactions may be impaired in smokers.

Smoking has also been shown to have a deleterious effect on periodontal wound healing. Preber and Bergstrom demonstrated that there was less reduction in periodontal probing depths in 40 heavy smokers after non-surgical periodontal therapy than in 35 non-smokers (206). It was also found that abstention from smoking allowed healing to return to normal levels.

1.4.3 Ageing and periodontal disease

Seymour and Heasman have extensively reviewed the effects of ageing on periodontal disease (207). Life expectancy has increased substantially during the course of the twentieth century, and it has been estimated that 45% of the overall aged population will be 75 years and over by the year 2000 (208). Five million of these in the U.K. will be 85 or over. This tendency towards increases in older and older cohorts has substantial impact on rates of periodontal destruction and treatment needs. For example, Fox *et al* (20) studied 554 people aged 70 to 96 and discovered substantially higher estimates of periodontal destruction among older age groups than previous studies have suggested. Their results suggest that, as older and

older age groups survive, rates of recorded periodontal disease increase as does demand for periodontal services.

There are changes in plaque composition with age. Spirochaete levels increase with age (209), but numbers of *Streptococci* decrease (210), suggesting increasing anaerobiasis with ageing. There are also changes in the periodontal structures. Alveolar bone shows marked changes with age. These include a decrease in the osteogenic layer of the cribriform plate. Moreover, the periodontal surfaces of the alveolar bone become jagged and collagen fibres show a less regular insertion into bone (211).

Thus, physiological and microbiological changes having a bearing on periodontal disease occur with ageing. Recently, defects in the specific humoral immune response with ageing have also been demonstrated (212,213). Part of this study, therefore, investigates differences between young and old individuals in terms of immune and inflammatory responses.

1.4.4 Potential relevance of oral tolerance to periodontal disease

The following discussion of the phenomenon of oral tolerance springs from the hypothesis that the immune system may be confused in its recognition of periodontal antigens because of their continual presentation to the gastrointestinal tract in a manner similar to that of food antigens. Lopatin *et al* (190) have speculated that *in utero* exposure to periodontal antigens may occur because of pregnancy gingivitis, which is often severe leading to bacteraemia (214-216), followed by placental transfer. Clearly, if this occurs early during immune maturation, there is the real possibility that these antigens will be recognised as auto- rather than allo-antigens. These two mechanisms of *in utero* exposure and oral tolerance may, indeed, account for the fact that the avidity of antibodies to periodontal antigens is generally very low compared with human responses to standard antigens, e.g. tetanus toxoid, or responses in animals vaccinated with periodontal bacteria (190). Therefore, an investigation was carried out within this study to establish whether or

not bacterial DNA could be detected by polymerase chain reaction (PCR) in the human bloodstream.

Oral tolerance refers to the observation that if one feeds a protein and then immunises with the fed protein, a state of hyporesponsiveness to the fed protein exists. It was first described by Wells in 1911 (217). Guinea pigs were fed hen's egg proteins, and were subsequently found to be resistant to anaphylaxis when challenged with the same proteins. The phenomenon has recently been confirmed in humans fed, and later immunised with keyhole limpet haemocyanin (218).

Immunological tolerance is a basic property of the immune system that provides for self/non-self discrimination so that the immune system can protect the host from external pathogens without reacting against self. When the immune system reacts against host antigens, autoimmune disease results. For a time it was thought that self/non-self discrimination was a simple matter of deleting autoreactive clones in the thymus, but it is now clear that the maintenance of immunological tolerance is a much more complicated process. Autoreactive cells, such as those reacting with brain, are not deleted and can be found in normal individuals

(219,220). Why these cells become activated and cause disease in some individuals, whereas in others they remain harmless is a major question in basic immunology. However, the mechanism of oral tolerance has been recognised as being so important that human trials involving oral administration of myelin basic protein (MBP) are now under way in patients with multiple sclerosis (221), who have auto-antibodies to MBP.

There are two primary mechanisms by which oral tolerance is induced, active cellular suppression or clonal anergy. Low doses favour active suppression, whereas high doses produce anergy (222-224). Active suppression is mediated by the induction of regulatory T-cells in the gut-associated lymphoid tissue (GALT), such as Peyer's patches. This mechanism would appear to be more relevant in periodontal disease, when low but constant doses are being experienced. There have been no studies of the relevance of oral tolerance to periodontal disease, but an interesting observation is that lipopolysaccharide (LPS) enhances oral tolerance to MBP (225).

1.5 Other clinical correlates which may have relevance to periodontal disease

1.5.1 Introduction

In this section other clinical correlates which may have relevance are discussed. Periodontal disease may also be a model system of immune-inflammatory disorders at the local level.

1.5.2 Rheumatoid arthritis

Mechanisms of bone resorption have been shown to be similar in periodontitis, rheumatoid arthritis and osteomyelitis (226). Lerner showed that haptoglobin, an acute-phase protein, can stimulate bone resorption *in vitro*, indicating the possibility of generalised bone loss in chronic inflammatory diseases. The fact that bone loss in periodontal disease is limited to the locus of inflammation may have implications for other disease processes which have systemic consequences. Inflammation-induced localised bone resoption in these immune-

inflammatory disorders is due to activation and recruitment of osteoclasts by locally produced cytokines and inflammatory mediators. Inflammatory bone resorption, which is a feature of both rheumatoid arthritis and periodontal disease, appears to be mediated by interleukin-1β (IL-1β) (227). This appears to happen via the induction of plasminogen activator activity converting plasminogen to plasmin, which in turn can activate precursor procollagenase to collagenase. B1 and B2 kinin receptors are also involved in inflammation, by e.g. causing release of prostaglandins, histamine and IL-1β.

This has led to speculation that B1 and B2 receptor antagonists allied with γ-interferon might serve as a pharmacological basis for more effective treatment of joint inflammatory and related diseases such as periodontal disease (228). Most interestingly, Jeffcoat et al have indicated that non-steroidal anti-inflammatory drugs (NSAIDs) may slow alveolar bone loss in periodontal disease, and they conclude that further studies of such methodologies are required to more fully investigate the effects of NSAIDs in rheumatoid arthritis (229). For these reasons, more consideration of the pathogenesis of

rheumatoid arthritis may be useful to our understanding of periodontal disease, which may be viewed as a model immune-inflammatory process at the local level.

Rheumatoid arthritis, like periodontal disease, seems to have a genetic basis. Stastny demonstrated that HLA-DR4 (one of the antigens of the major histocompatibility complex (MHC)) was associated with an approximately fourfold relative risk of rheumatoid arthritis in North American populations (230). However, in certain sub-populations, rheumatoid arthritis is more often associated with DR1 than DR4 (231). Using molecular biological techniques, McDaniel et al were able to detect one fragment of β-chain DNA produced by one endonuclease that appears to carry with it a 50-fold increased susceptibility for rheumatoid arthritis (232).

Aetiological factors which have been suggested include Epstein-Barr virus (233) and parvoviruses (234). However, the discovery that Helicobacter pylori is involved in gastrointestinal ulceration suggests that a bacterial aetiology cannot be ruled out for diseases like rheumatoid arthritis. Moreover, Girouard et al have shown that there is an association between the presence of autoreactive antibodies against the recombinant human heat

shock protein, huHsp60, and infection with the spirochaete, *Borrelia burgdorferi* (235). Heat shock proteins are highly conserved, and it is suggested that exposure to microbial Hsp60 stimulates the production of auto-antibodies to huHsp60. The potential importance of spirochaetes in periodontal disease will be discussed in section 1.6., but difficulties in culturing these organisms has restricted data in both disease processes. However, the ability of these organisms to invade tissue may have implications for the humoral immune response.

In a comparative study of patients with rheumatoid arthritis and periodontal disease, Macey et al found generalised expression of adhesion molecules on peripheral blood leucocytes from rheumatoid arthritis patients but not periodontitis patients, suggesting that localisation of inflammatory activation occurs in periodontal disease (236). Zheng et al demonstrated that platelet activating factor (PAF) may mediate osteoclastic resorption in bone and mineralised cartilage in both rheumatoid arthritis and periodontal disease (237).

Recent work on anti-TNF-α monoclonal antibody as a therapy for rheumatoid arthritis may have considerable implications for periodontal disease treatment (238).

Over-production of TNF-α has been shown to promote inflammation by stimulating the further over-production of IL-1β, IL-6, IL-8 and granulocyte/macrophage-colony stimulating factor.

1.5.3 Pregnancy

Normal pregnancy involves immunological mechanisms allowing tolerance of paternal antigens by the mother. Rocklin et al showed that maternal lymphocytes can produce an immigration inhibitory factor in normal pregnancy (239). Their studies on the nature of the blocking factor indicated that this factor was an IgG antibody and it was directed against paternal antigens. Moreover, women who abort lack this antibody in their sera (240-242). It has been suggested that recurrent miscarriage is in many cases related to immunological factors (243). Previous studies have shown that IgG and IgM production are significantly raised in the first trimester of normal pregnancy (244-246). In addition, miscarriage has been shown to be associated with increased incidence of thyroid auto-antibodies (247-249).

These findings suggest that normal pregnancy involves the production of an IgG inhibitory antibody, and miscarriage involves production of auto-antibodies and anti-paternal-antigen antibodies. These possibilities were thought worthy of further investigation within this study. Moreover, by PCR analysis detection of bacterial DNA could be attempted in the bloodstream to establish if bacterial antigens can cross the placenta thus inducing tolerance of oral bacteria, as discussed in section 1.6.

1.6 Specific Humoral Immune Response in Periodontal Disease

1.6.1 Introduction

When we come to consider the specific humoral immune response in periodontal disease, i.e. antibodies directed against particular oral micro-organisms, there are a number of issues which must be addressed. First, the organisms to which a response is being detected must be considered. At this point microbial aetiology and pathogenesis must be taken into account since, as has previously been discussed, organisms may provoke an immune response but not fulfil other aspects of the extended postulates (179). In addition, antibodies may be detected to non-oral bacteria and non-bacterial antigens (86,87). Some investigators have used a battery of micro-organisms, including many of doubtful relevance, and this has led to an apparent lack of focus. In this study *P. gingivalis* and *A. actinomycetemcomitans* were chosen for particular attention because of their strong association with

periodontal disease using all of the criteria previously mentioned.

Secondly, there has been controversy over whether whole bacterial cells should be used in assays of specific antibody, or particular antigens isolated from these cells (250). However, in this study, it was decided to generally employ whole cells because of the importance attached to another aspect of the antibody response, that of antibody avidity. This can be seen as a global factor made up of the strength of interactions between numerous bacterial antigens and numerous host antibodies produced in response to these. Therefore, the overall response to a particular bacterium may yield information that would be lost by strict concentration on specific antigens, which may, in fact, not be virulence factors.

Thirdly, and related to the last point, there is the matter of antibody function. Clearly, demonstrations of an association between host antibody response and periodontal disease may be only academic unless it can be related to the biological function of this antibody. These functions, which include the ability to opsonise bacteria and the ability to bind strongly to fimbriae,

which may prevent bacterial colonisation, may relate to antibody avidity.

Fourthly, we must consider whether local antibody levels in the GCF or systemic levels or both are of importance; and whether local levels are merely a reflection of serum levels, or whether significant antibody production by gingival plasma cells is taking place. This is important in the consideration of subject and site susceptibility to disease onset and progression.

Fifthly, assessments of the titre and avidity of patient's antibody to suspected periodontopathogens may be useful in the differential diagnosis and classification of periodontal diseases.

Sixthly, the assay of these antibodies in longitudinal studies may provide information on the relationship between antibody titre and avidity and disease progression at both subject and site levels, which may prove to be a marker of disease activity.

Finally, there is evidence that the subclass of immunoglobulin produced has a bearing on aspects of its function such as complement fixation and opsonisation.

1.6.2 Response to Gram-positive Organisms

Most studies concerned with the humoral immune response to oral micro-organisms have tended to concentrate on Gram-negative organisms, but there have been some which have focused on Gram-positive organisms; however few of these have been in recent years.

An earlier study by Ebersole's group assayed IgG and IgM antibody directed against *Actinomyces naeslundii*, *Actinomyces viscosus*, *Eubacterium brachy*, *Streptococcus mutans* and *Streptococcus sanguis* in localised juvenile periodontitis (LJP), generalised juvenile periodontitis (GJP), adult periodontitis (AP), acute ulcerative gingivitis (AUG), and edentulous patients and normal subjects. For all of these bacteria and all of these disease states there was no difference from the normal subject population (251). Similarly, this group went on to examine antibody titres to some of these organisms in Papillon-Lefevre syndrome, a type of neutrophil dysfunction manifesting with severe periodontal disease (252). In this study no significant response to these organisms was detected. This does not suggest an important role for such organisms in this disease

aetiology, since it might be expected that if Gram-positive antigens were penetrating the gingival tissues they would provoke a humoral immune response.

Page and Ebersole's groups also collaborated in a study which assayed levels of antibody to *Actinomyces naeslundii* in a family with a high prevalence of juvenile periodontitis (JP) (253). Elevated IgG levels to this organism were not detected although both patients and controls showed high levels of *Actinomyces* species in the subgingival microflora.

Ebersole's group have concluded that relatively abundant constituents of the Gram-positive flora in subgingival plaque, e.g. *Actinomyces* species, *Streptococcus mutans* and *Streptococcus sanguis*, do not provoke a significant humoral response even in patients whose microflora contain a large proportion of these organisms (254). They have also shown that these bacteria do not contain antigens that cross-react with Gram-negative constituents of the plaque (251,255).

Studies of these organisms by other groups have also failed to demonstrate increased titres in periodontal disease (256-258). These have generally shown wide variation, lack of discrimination between diseased and

healthy groups, and, in one study (256), higher levels of IgG and IgA antibody to *Streptococcus sanguis* in healthy controls than in periodontitis sera. The only exception seems to be a study in 1970 by Nisengard and Beutner (259) showing higher levels of IgG antibody to *Actinomyces* species with increasing periodontal inflammation. However, the antibody assays in this study were by immunofluorescence and, therefore, the facility for quantification was probably inferior to that achieved in later ELISA-based studies.

Generally, differences in the humoral immune response to Gram-positive bacteria do not appear to occur between periodontitis patients and healthy controls. However, a notable exception may be the study of Tew *et al* (260) which examined antibody levels to *Eubacterium* species, *Lactobacillus minutus* and *Peptostreptococcus micros*; this being based on their previous study (261) in which they showed these organisms to be predominant members of the cultivable subgingival microflora in a cohort of patients with severe periodontitis. Their findings were that IgG antibody to *Eubacterium brachy* and *Peptostreptococcus micros* was raised in those patients but not in JP patients or healthy controls. However, it must be borne in mind

that this study employed radioimmunoassay (RIA), rather than ELISA, which is known to be more sensitive, and refers to dichotomous measures of seropositivity/seronegativity rather than quantitative differences.

Therefore, it seems fair to conclude that there are no abnormal changes in the humoral immune response to the Gram-positive flora in periodontal disease; but that constituents of this flora, e.g. *Eubacterium* species, although probably not aetiologic, may assume a more predominant role during the change to a pathogenic flora. There may concomitantly be an antibody response which could be a useful marker of this process.

1.6.3 Response to Gram-negative Organisms

Studies of the humoral immune response to Gram-negative organisms have tended to target organisms such as *P. gingivalis* and *A. actinomycetemcomitans* because of their marked association with periodontal disease (164). An earlier study of particular relevance is that of Mouton *et al* in which serum IgG, IgM and IgA antibody to *P.*

gingivalis was assayed in various patient groups, including AP, LJP and GJP (or RPP), and also in healthy controls (262). This report established that detectable levels of antibody to *P. gingivalis* are found in a significant proportion of healthy adults and also that there is a positive correlation between antibody levels and age. 80% of the healthy individuals studied had IgG antibody to this organism, 55% had IgM and 34% had IgA. Moreover, IgG and IgM serum antibodies were detectable in children as young as six months, and children aged 6-12 demonstrated significantly higher antibody levels than in younger children. The authors concluded that this organism probably has an aetiologic role in the disease process, and that the antibody response is essentially protective.

In addition, mean levels of IgG antibody were found to be five times higher in AP patients and eight times higher in RPP patients than in controls. Higher IgM levels were seen in JP patients and higher IgA levels in RPP. Therefore, this paper was important in establishing a number of points:-

1. Based upon the humoral immune response, *P. gingivalis* is probably aetiologic in periodontal disease.

2. This response is probably protective.

3. Diseased and healthy individuals can be distinguished in terms of their antibody response to this organism.

4. There are indications of differences in the response in different periodontal disease states.

These findings were confirmed and extended by this and other research groups. Ebersole *et al* (263) studied 85 AP patients, 67 JP patients, 62 RPP patients and 82 controls, and found that 95% of JP patients had elevated antibody levels to at least one organism, predominantly *A. actinomycetemcomitans*. 48% of AP patients, and 37% of RPP patients had elevated serum antibody levels to *P. gingivalis*, whereas 85% of control subjects showed no elevations in antibody to any of the organisms in their battery.

Naito *et al* (264) assayed IgG antibodies to *P. gingivalis* in 42 AP patients and 17 controls. They graded the AP patients according to disease severity and found a positive correlation of antibody level and disease severity. Although antibodies to *A. actinomycetemcomitans* were also elevated in this study, this was not significant, and, therefore, this was perhaps an

indication of the differing relevance of these two organisms in AP.

1.6.4 Response to *Porphyromonas gingivalis*

Two studies by Ebersole *et al* (255) and Tew *et al* (260) examined antibody responses to various members of the *Bacteroides* genus as it was then classified. These included *Bacteroides gingivalis*, now *Porphyromonas gingivalis*, and *Bacteroides intermedius*, now *Prevotella intermedia*. Although elevated responses to other *Bacteroides* species were detected in some periodontitis patients, especially to *Prevotella intermedia*, *P. gingivalis* was found to be the most consistent example of a *Bacteroides* species eliciting an antibody response during periodontal disease; these responses being most frequent and of greatest magnitude.

Moreover, an investigation by Gmur *et al* (265) reported that IgG antibody levels to *P. gingivalis* were highly correlated with the extent of periodontal destruction, whereas anti-*Prevotella intermedia* reactivities were not. Similarly, studies by other groups

have confirmed that antibody levels to *P. gingivalis* are higher in AP patients than in controls (266,267).

Although these studies have tended to concur that an increased antibody response to *P. gingivalis* is associated with periodontal disease and may serve as an indicator, the protective nature of this antibody has been questioned. For example, Tollefsen *et al* (268) reported that immunoglobulin levels to *P. gingivalis* were lower in immunosuppressed transplant patients than in periodontitis patients, although the immunosuppressed patients also had a lower frequency of bleeding on probing and lower mean pocket depth than the periodontitis patients. They concluded that a reduced immune reactivity to plaque bacterial antigens leads to a milder and less destructive form of periodontitis. However, since both patient groups were not shown to be comparable in terms of previous periodontal disease experience, and the effects of immunosuppression on inflammation and vascularity could not be ruled out, it is difficult to see how such a conclusion can be drawn.

Generally, reports in this area have agreed that there is a positive relationship between serum antibody to *P. gingivalis* and other suspected periodontopathogens and

periodontitis. However, there have been a significant number of studies reporting no difference between patients and controls or even lower antibody levels in patients (256,260,269-273). Although some of these studies are not particularly recent, and, therefore, may be seen as less accurate in their antibody assays, others, such as that of Chen *et al* (273) present a more significant counterpoint to this argument. These workers studied RPP in particular and found that some patients mounted a humoral immune response to *P. gingivalis*, whereas others did not. In addition, these antibodies were of low avidity, but periodontal therapy could induce the production of higher avidity, and presumably more biologically effective antibodies. Thus, Chen *et al*, in their discussion, crystallise many of the issues which will be dealt with in this study in relation to the humoral immune response to *P. gingivalis*, e.g. patient susceptibility, diagnostic categories, treatment effects, and the importance of antibody avidity.

Recently, Gemmell et al have demonstrated that gingivitis and periodontitis subjects with *P. gingivalis* infection may recognise different antigens. More specifically, periodontitis patients' sera reacted against

five lower molecular weight antigens falling within the range of lipopolysaccharide (LPS) antigens, whereas gingivitis patients' sera reacted against four higher molecular weight antigens. There was a significantly higher recognition of the 91.4kDa band in gingivitis than in periodontitis sera (274).

Wheeler et al have demonstrated that serum IgG levels to *P. gingivalis* were positively correlated with loss of alveolar bone in an elderly population, whereas antibody levels to *F. nucleatum* and *P. intermedia* were negatively correlated (275). Nakagawa et al found a positive correlation between serum IgG levels to *P. gingivalis* and homologous infection (276).

1.6.5 Response to Actinobacillus actinomycetemcomitans

An early study of the antibody response to *A. actinomycetemcomitans* linked this response specifically to LJP by demonstrating the presence of these antibodies in these patients but not in normal subjects (277). Ebersole *et al* also showed an association between increased levels of,

and increased frequency of, antibody to *A. actinomycetemcomitans* and LJP (278). These authors elaborated on this in a later study (279) in which they showed a significantly increased level of IgG antibody to *A. actinomycetemcomitans* serotype b in 90% of LJP patients, but only 40% of RPP and 25% of AP patients. Listgarten *et al* (280) had previously made similar findings by immunofluorescent techniques. Several other reports have shown a strong association between antibody to *A. actinomycetemcomitans* (especially serotype b) and LJP (271,281, 282).

Zambon, in his review of 1985, stated that *A. actinomycetemcomitans* was an important organism in the aetiology of JP because of its increased prevalence in LJP patients and their families, and increased antibody responses to this organism (139).

Eisenmann *et al* (283) reported similar results with a Central African population and suggested that susceptibility to JP may have a genetic component related to a gene pool originally derived from Africa. This hypothesis was tested by a study examining the effect of race and periodontal status on antibody to *A. actinomycetemcomitans* (284). This showed a relationship between

high levels of antibody to *A. actinomycetemcomitans* Y4 and both diagnosis of JP and black race.

Other studies in other ethnic groups have confirmed that *A. actinomycetemcomitans* is frequently associated with, and probably an aetiologic agent of JP, and may also be implicated in certain cases of RPP (285,286). In addition, an investigation of anti-*A. actinomycetem-comitans* antibodies in individuals with AP showed no correlation between antibody levels and disease (265).

Recently, Wilson et al have shown that patients with localised juvenile periodontitis (LJP) produce IgG2 antibodies directed against the outer membrane proteins of *A. actinomycetemcomitans* which promote opsonophagocytosis of *A. actinomycetemcomitans* (287,288). O'Dell and Ebersole hypothesised that antibody avidity to *A. actino-mycetemcomitans* could help to explain the relationship between the active host response and chronic infection with this pathogen. Their data suggested that both antibody levels and avidity may contribute to the variation in host resistance to infection and disease associated with *A. actinomycetemcomitans* (289). Underwood et al have demonstrated that anti- *A. actino-mycetemcomitans* antibodies are important in promoting

phagocytosis and killing of *A. actinomycetemcomitans*. They suggest that subjects who develop high levels of highly avid antibodies against *A. actinomycetemcomitans* may have greater resistance to continued and repeated infection by this pathogen (290).

1.6.6 Response to other Gram-negative Organisms

Antibody responses to other Gram-negative organisms have generally failed to demonstrate any consistent and convincing association between these responses and the occurrence and extent of periodontal disease. For example, Ebersole *et al* could not demonstrate any association between antibody levels to the *Capnocytophaga* species, *gingivalis*, *ochracea* and *sputigena*, and particular disease classifications (291,292). Levels of antibody to *Fusobacterium nucleatum* have been shown to decrease after the age of twelve years in children with insulin-dependent diabetes (293), in contrast to the previously reported positively age-related antibody levels to *P. gingivalis*. *Eikonella corrodens* has been shown to provoke an antibody response during mono-infection

induced periodontitis in gnotobiotic rats (294,295). However, such studies are of doubtful relevance to human periodontal disease, not only because of the animal model employed but also because human periodontal disease involves mixed plaques rather than single organisms. It may be that some organisms, although capable of provoking periodontal destruction under artificial conditions, are normally limited in their effects by other plaque flora.

1.6.7 Response to Spirochaetes

There have also been some studies of antibodies directed against spirochaetes. Generally, these have not shown significant differences in antibody responses to organisms such as *Treponema denticola* between patients with periodontal disease and healthy controls (260,296-298). Four explanations have been advanced to account for these findings:-

1. Spirochaetes lack antigenicity.
2. Immunosuppression by spirochaete products.

3. Spirochaetal colonisation is limited to subgingival plaque. Organisms and their products do not pass into host tissues.

4. The quality of culture techniques for spirochaetes is insufficient to inspire confidence in derived ELISA systems.

However, spirochaetes have been shown to be antigenic in animals, and variable responses in the above studies demonstrate their antigenicity in humans. Also, immunosuppressive agents produced by spirochaetes have been shown to be generally suppressive *in vitro*. Therefore, the most probable explanation is that spirochaetes are limited to the subgingival plaque. The implication that spirochaete products cannot invade host tissues, although whole spirochaetes have been shown to invade the gingiva to a limited extent in AUG (170), does not suggest an aetiologic role. Spirochaetes have, however, been shown to be particularly associated with AUG. Even in this condition, however, no significant difference was seen in antibody levels to Borrelia vincentii between patients and controls (299).

1.6.8 Response to Particular Antigens

Some studies have investigated the humoral antibody response to particular antigens of bacteria rather than whole bacterial cells. However, intensive research into the fimbriae of Gram-positive organisms such as *Actinomyces viscosus* (300-304), on the premise that anti-fimbriae antibodies could block adherence and thus colonisation, do not appear to have taken into account the extensive literature showing that no significant response to these organisms occurs in periodontal disease. Similarly, a study showing differences in the ability of antibodies of various inbred strains of mice to bind to fimbriae of *Actinomyces viscosus* (305) is of doubtful relevance for the same reason, and also because of the equivocal contribution of such animal models to our understanding of the aetiology and pathogenesis of periodontal disease in humans.

Increased levels of IgG antibody to LPS of *P. gingivalis* have been demonstrated in periodontitis patients (251,306). Another study demonstrated increased antibody levels to trypsin-like protein produced by *P. gingivalis* in periodontitis patients compared with control

subjects (307). However, the ability of this antibody to inhibit this enzyme has not been shown.

There have also been investigations of the antibody response directed against fimbriae of *P. gingivalis*. Mouse monoclonal IgG1 antibody developed against *P. gingivalis* strain 381 inhibited adherence of *P. gingivalis* to buccal epithelial cells (308). Further work has shown that 67% of AP patients had antibody reactive with fimbriae, and that antibody activity could be detected to the 43kDa fimbrillin monomer and also to dimers, trimers and oligomers (309).

Ebersole *et al* have demonstrated differences in the antibody responses to particular membrane antigens of *P. gingivalis* (310). However, it should be noted that these antigens have not been identified as virulence factors, and patients with strong responses to these antigens often tended to be those who had a strong response to whole cells. Although these studies will undoubtedly help to elucidate the immune response to *P. gingivalis*, their use in clinical studies can be criticised on three points:-

1. Until particular antigens are identified as virulence factors, the demonstration of antibodies against these may be of doubtful relevance.

2. The results of some of these studies, e.g. that of Watanabe *et al* (311), have suggested that antibody reactivity to particular groups of antigens may be no more useful than to whole cells.

3. Focusing too narrowly on particular antigens may result in other valuable information being lost.

A study of antibody directed against LPS of *A. actinomycetemcomitans* found no increase in AP or JP patients over controls (312). By contrast, an investigation of the ability of antibody to neutralise *A. actinomycetemcomitans* leucotoxin found that 95% of JP patients demonstrated these antibodies as compared with much lower frequencies in other patient groups and control subjects (313).

Other studies have also dealt with antibodies directed against leucotoxin (314,315). However, the association between these antibodies and JP can also be shown in studies of antibodies to whole *A. actinomycetemcomitans* cells (285,286), and the relevance of leucotoxin

as a virulence factor has not yet been demonstrated *in vivo*.

Recently, Aduse-Opoku et al have characterised a cell surface or extracellular arginine-specific protease antigen which has previously been shown to have significant reactivity with IgG antibody of periodontal disease patients (316). Since these proteases have previously been shown to give *P. gingivalis* the ability to degrade human proteins (145), a study of the serum antibody avidity was included as part of this investigation. The aim of this was to determine if this protease is a virulence factor, and to further elucidate the relationship between the systemic response to this antigen and disease progression. It has also been suggested recently that it may be possible to distinguish gingivitis from periodontitis patients in terms of patterns of response to outer membrane antigens of *P. gingivalis*. Gemmell et al found that gingivitis patients had significantly more antibody to a 91.4kDa antigen and significantly less antibody to LPS than periodontitis patients (274).

1.6.9 Antibody Avidity

Antibody avidity, that is a measure of the net binding strength between antibodies and antigens, has been extensively studied in a number of fields, both in relation to antibody titre and in isolation, in terms of disease susceptibility and progression. For example, Morikawa *et al* (317) found that the titre of IgA to soybean antigen in the breast milk of Indian mothers was significantly higher than in Japanese mothers, although the avidity was significantly lower. Udhayakumar *et al* (318) have shown that monoclonal antibodies of higher avidity have much greater effectiveness in activating B-cells and presumably enhancing an immune response. Interestingly, Charoenvit *et al* (319) found that a monoclonal antibody to *Plasmodium yoelii*, a malarial parasite, had lower avidity for the antigen than vaccine-induced polyclonal antibodies in mice.

Doi *et al* (320) suggested that low avidity may be a pathogenic characteristic of IgG circulating immune

complexes in membraneous nephropathy. Significantly, Joynson *et al* (321) found that toxoplasmosis patients with acute infection had low avidity IgG to this organism whereas those with chronic infection had high avidity. This may have application in investigations of the phasic destructive episodes of chronic periodontitis, with the possibility that acute exacerbations and quiescent periods could be differentiated.

The secondary immune response classically generates antibodies of higher avidity. Francus *et al* (322) have demonstrated that a possible mechanism may be carrier primed T-cells which selectively activate virgin B-cells, which are then committed to the production of high avidity antibodies. A study with particular relevance to the suggested autoimmune aspects of periodontal disease is that of Panoskaltsis and Sinclair (323), who showed that autoimmune mice have lower avidity anti-single strand DNA than non-autoimmune mice.

There have, however, been very few reports dealing with antibody avidity in relation to periodontal disease. Ebersole *et al* (324) studied the increase in avidity in the non-human primate, *Macaca fascicularis*, following immunisation with tetanus toxoid, which they used as a

prototype bacterial exotoxin. They found that IgG avidity increased from 0.9M to 1.72M following primary immunisation, and 2.56M after secondary immunisation. Lopatin *et al* (190) demonstrated that avidity of antibody rose to a similarly high level in rabbits post-immunisation with *P. gingivalis*, but that human antibodies to this organism appear to be of generally low avidity. They also discovered that IgG antibodies to *P. gingivalis* were of significantly higher avidity in periodontitis patients than in control subjects (0.96M compared with 0.71M). They suggested that the presence of low-avidity antibodies contributes to the pathology associated with periodontal disease.

Chen *et al* (273) demonstrated that IgG avidities to *P. gingivalis* were lower in RPP patients than in control subjects. However, after treatment, the avidities increased significantly to levels higher than in controls. They concluded that many RPP patients do not produce protective levels of biologically functional antibody as a result of natural infection, but that treatment may induce the production of such antibodies. Another recent study of titre and avidity of IgG antibodies to *P. gingivalis* in RPP patients by Whitney *et al* (325) also shows lower

avidities in RPP patients than in controls. A recent study by Sjostrom *et al* (326) showed that IgG antibodies in low-titre sera from control subjects were significantly more effective in opsonising *A. actinomycetemcomitans* than IgG antibodies in low-titre sera from RPP patients. This study suggests a crucial link between antibody avidity and function. Further support for this is derived from two recent reports demonstrating that effective binding of IgG to virulent *P. gingivalis* has a crucial role in complement activation and also in the opsonisation and phagocytosis of this organism (327,328).

A recent study by Ebersole and Kornman (329) demonstrated that *P. gingivalis* emerges as an organism in the subgingival plaque during the conversion from gingivitis to progressing periodontitis in a non-human primate model, and that this elicits a systemic antibody response specific for this organism. Similarly, a study by Dahlen and Slots (142) in rabbits showed that animals co-inoculated with *P. gingivalis* and *A. actinomycetem-comitans* showed significantly more severe disease than animals which were monoinfected. They concluded that the immune system acting through systemic antibodies and/or cellular mechanisms may modulate the pathogenic potential

of infecting periodontal pathogens. It may be that antibody avidity has a crucial role in this modulation.

A recent study in this laboratory showed that chronic periodontitis patients who went on to develop further attachment loss, in a longitudinal study, had lower systemic antibody levels to *P. gingivalis* than those who did not (330). This was statistically significant. This finding was confirmed by O'Dell and Ebersole in terms of the avidity response to *A. actinomycetemcomitans* (289). Their study also suggested a potential prognostic value for avidity measurement in destructive periodontal infection. They found that, in patients who were positive for *A. actinomycetemcomitans* infection, avidity values tended to decrease as clinical parameters worsened and infection increased.

1.6.10 The Local Response

Most studies investigating the humoral immune response have concentrated on systemic antibody levels. Much less work has been done on the relationship between local antibody levels and local disease status. Baranowska *et*

al (331) found no significant difference in the level of specific IgG to *P. gingivalis* in GCF between healthy and diseased sites within the same individual. Tew *et al* (332) found no obvious differences in the clinical parameters of probeable depth and attachment level between sites with elevated antibody to *P. gingivalis* and/or *A. actinomycetemcomitans*, and those with normal or low levels, and concluded that elevated antibody in GCF may relate to changes in disease activity that are not detectable by normal clinical assessments.

However, Suzuki *et al* (267) demonstrated that local production of IgG to *P. gingivalis* was markedly increased in AP as compared with RPP patients, suggesting that disease progression is influenced by local antibody production. Challacombe *et al* (333) showed that levels of IgG antibody to *P. gingivalis* were lower in crevicular fluid washings (CFW) of patients with high periodontal disease index (PDI) as compared with those with low PDI, although this was not found to be statistically significant. Opsonic activity against *P. gingivalis* was, however, found to be significantly depressed in high PDI as compared with low PDI patients.

Lamster *et al* (334) reported a significant correlation between total IgG in GCF and specific serum antibody to *Prevotella intermedia* but not *P. gingivalis.* They concluded that the development of a serum immunoglobulin response to suspected periodontopathogens is consistent with a protective host response. A corollary of this view is that a local deficiency of IgG to these pathogens may lead to local disease progression. Alternatively, Kilian (145) has demonstrated that *P. gingivalis* can degrade human IgG and IgA, suggesting that low GCF levels of IgG may be caused by degradation by this organism, or that locally available antibodies are adsorbed by the greater mass of subgingival plaque present.

An earlier study by Schenck (335) showed an inverse relationship between the number of deep pockets (>4mm) and serum antibody level to *P. gingivalis* LPS in chronic periodontitis patients. Similarly, Mouton *et al* (336) demonstrated a dichotomy in serological responses to *P. gingivalis* among chronic periodontitis patients, with one subgroup exhibiting high serum antibody levels and another having levels similar to those of healthy individuals.

Jansen et al have recently adduced further evidence suggesting that the ability of *P. gingivalis* and other oral bacteria to degrade host IgG may be important in the causation of polymicrobial infections (337).

It has been previously demonstrated in this laboratory that lower GCF antibody levels to *P. gingivalis* were found in deeper pockets and in more inflamed sites (338). Recently, OuYang has corroborated this by showing that periodontitis patients had lower GCF levels of antibody to *P. gingivalis,* when related to serum levels, than gingivitis patients (339). OuYang had previously demonstrated that elevations in the GCF/serum ratio of antibody to *P. gingivalis* occurred one month after periodontal treatment (340). This suggested that local antibody consumption may be reduced after removal of the organism and that the GCF/serum ratio of antibody level might be used as a significant indicator in evaluation of treatment effectiveness. Moreover, it has also been established in this laboratory that IgG levels were lower in inflamed tooth and implant sites than in healthy matched sites (341).

Moreover, the finding of Haffajee et al that patterns of response are important in disease differentiation in

periodontitis, suggests that local patterns of response in the GCF may also be of interest. Therefore, a feasibility study was carried out using GCF from patients with early-onset periodontitis using checkerboard immunoblotting. This allows patterns of response to a number of species and strains, particularly serotypes of *A. actinomycetemcomitans*, to be assessed qualitatively.

1.6.11 The Host Response in Differentiation of Disease States

The humoral immune response has been suggested as a means of differentiating between distinct periodontal disease states. A strong local and systemic antibody response to *A. actinomycetemcomitans* has been adduced as an indicator of JP (342-344). An association between the most severe and extensive cases of RPP and relative lack of antibodies to *P. gingivalis* and/or *A. actinomycetemcomitans* has also been reported (271,345). This, coupled with the previously quoted findings that humoral responsiveness in RPP in terms of antibody avidity is depressed compared

with that found in AP patients and healthy individuals, suggests possible disease mechanisms. When localised and generalised early-onset periodontitis (EOP) cases (i.e. JP and RPP) are grouped together and the extent and severity of disease is related to age and plaque index, there is a positive correlation for the RPP group for both factors. This contrasts with the JP group for which the correlation is significant for plaque only (346). This would be consistent with an interdiction of disease progression in JP by an effective immune response, while those with an ineffective response would go on to develop generalised disease (RPP). Previous hypotheses have connected JP with PMN abnormalities (95-97), but these findings have been challenged recently (98-100). Thus this area would appear to be one in which fruitful progress may be made in enhanced differential diagnosis by objective tests of the humoral immune response.

It has recently been shown in this laboratory that adult periodontitis (AP) and rapidly progressive periodontitis (RPP) patients can be distinguished in terms of their avidity responses to *P. gingivalis*. RPP patients were found to have significantly lower avidities than AP patients (347). Haffajee et al have also been able to

show differences in patterns of antibody response in differing periodontal disease groups (348).

The most striking feature of early-onset periodontitis patients in this study was elevated serum antibody levels to *A. actinomycetemcomitans* serotype b. By contrast, adult periodontitis patients in this study were characterised by high levels of antibody serotype a. By methods based on those of bacterial taxonomy, the authors employed cluster analysis to assign patients to disease groups on the basis of patterns of antibody response. They found only responses to *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans* serotypes a and b were useful for this purpose.

1.6.12 Longitudinal Monitoring

In the most definitive study to date of longitudinal monitoring of humoral immunity in patients with destructive periodontal disease, Ebersole's group (349) studied 51 subjects bi-monthly for a period of up to five years. These comprised four subject groups which they

defined as AP, LJP, GJP and RPP. They found that, in general, most serum antibody levels to subgingival species remained relatively constant for periods of up to five years. However, major increases and decreases in antibody to specific species could be detected in certain subjects. They concluded that major changes occurring in serum antibody may reflect fluctuations in the nature of the infection. In addition, they stated that their data suggest that serum antibody levels to certain species over certain thresholds indicate an increased likelihood of disease activity taking place at some site in the oral cavity, and that it would be desirable to measure antibody to that species at the local sites in order to detect areas of active disease.

They further note differences between diagnostic categories and observe that the pattern of antibody fluctuation detected does not indicate a primary response, and that original antigen exposure probably occurred a long time in the past. This accords with the view of Lopatin *et al* (190) that immunologic tolerance may be induced by asymptomatic bacteraemia associated with often severe pregnancy gingivitis (214-216), resulting in *in utero* exposure, and leading to depletion of high-affinity

antibody-producing clones, leaving only low-avidity capability (350).

Horibe et al have recently demonstrated that mean antibody titres to *P. gingivalis* and *P. intermedia* decreased significantly after treatment (351). Their results suggest that the changes in serum IgG titres to these organisms are related to the suppression of these organisms in the subgingival plaque.

A longitudinal study of periodontal treatment effects is included as part of this study in order to elucidate the role of the humoral immune response in disease resolution.

1.6.13 Immunoglobulin Subclass

A small number of studies have focused on the subclass of IgG which is produced against suspected periodontopathogens. Reinhardt *et al* studied total IgG subclass levels without investigating the specificity of these antibodies (85). IgG1 and IgG4 levels were found to be higher in active as compared with stable periodontitis sites with similar clinical characteristics. A more recent

study investigated total IgG subclass levels in periodontitis patients and matched controls (352). This found that IgG2 levels were significantly higher in patients than in controls, and these data suggested that the predominant antibody response to periodontal pathogens is directed against carbohydrate or glycolipid antigens.

Whitney *et al* (325) investigated the titre, avidity and subclass distribution of serum IgG antibodies to *P. gingivalis*. They found the subclass order in both patients and controls to be IgG2>IgG3>IgG1>IgG4. They concluded that their findings were consistent with the hypothesis that both carbohydrate and protein antigens are important in the IgG response to *P. gingivalis*. However, the relative predominance of IgG2, a subclass which lacks strong complement fixation and opsonic properties, and the low avidity of patient anti-*P. gingivalis* antibodies suggested that the humoral response to infection may be ineffective in clearing this organism.

Lopatin and Blackburn (353) have recently reported that adult periodontitis patients produced IgG antibodies to streptokinase (SK) and tetanus toxoid (TT) with much higher avidity than to *P. gingivalis*. However, when the IgG subclasses were examined, the IgG1 antibodies to this

organism were found to be of similarly high avidity to those against SK and TT. Since IgG antibodies to *P. gingivalis* tended to be of the IgG2 subclass, which were of significantly lower avidity, the net effect of this predominating subclass was to lower the avidity of the overall IgG response to this organism.

Wilson and Hamilton have recently established that the concentration of IgG2 antibody to a major outer membrane protein of *A. actinomycetemcomitans* was higher than that of IgG1 antibody directed against this protein in seven out of fourteen LJP patients' sera. This response could be attributed to the presence of IgG2 antibodies to LPS or to glycoprotein. Therefore, these authors have shown that the IgG2 response to oral bacteria is not necessarily directed against carbohydrate or glycoprotein antigens (354). Wilson et al have also shown that these IgG2 subclass antibodies are opsonic for *A. actinomycetemcomitans* (355). Polak et al studied IgG subclass responses to outer membrane antigens (OMA) of *P. gingivalis* and discovered that IgG2 was the predominant subclass in terms of the number of OMA recognised, followed by IgG3, IgG1 and IgG4 respectively (356).

1.6.14 Vaccine development

The idea of a vaccine to control oral bacterial infections was developed in the early part of this century. Autogenous vaccines (357), pure cultures (358-359) and mixed stock vaccines (360-362) were all employed at various times. However, these approaches also had their critics (363-364). Recently, possible means of vaccine development have again been explored. Bird et al (365) used the mouse abscess model to show that protective immunity to *P. gingivalis* promoting resolution of lesions could be produced by immunisation with an outer membrane preparation of *P. gingivalis*. The depletion of CD4 T-cells prior to immunisation resulted in depressed serum levels of anti- *P. gingivalis* antibody and increased physical signs of disease. These findings suggested the protective role of serum antibody and a potential role for T-cell mechanisms in the control of the lesion locally. Page has recently reviewed the literature pertaining to the humoral response and its relevance to treatment response and vaccine development. The fact that the normal humoral immune response which occurs in patients with infectious disease, and that eventually arrests the

process of the infection, does not appear to happen in periodontal disease raises the question anew of human vaccine development as a means of addressing this problem (366).

1.6.15 Local plasma cell involvement

Seymour et al have proposed a model of the immunopathogenesis of chronic inflammatory periodontal disease. This suggests that susceptible subjects may have an increase in type 2 IL-4 producing T-cells selectively homing to the gingiva and leading to B-cell expansion. This may trigger periodontal destruction via IL-1 or protection via production of specific antibody by antigen-specific B-cells. Resistant subjects may have an increase in type 1 IL-2/IFN-α producing T-cells selectively homing to the gingiva resulting in B-cell suppression and a T-cell stable lesion with slow tissue destruction (367). Kinane et al used PCR amplification of the V-J junctions of T-cell receptor γ (TCRγ) gene rearrangements to establish that T-cells localising in gingiva differ from those in skin and peripheral blood. They conclude that

their findings are consistent with the existence of local immune systems composed of skin-homing and gingiva-homing memory T-cells (368). Shimauchi et al, in an adoptive transfer experiment, demonstrated that accumulation of a Th2 type *A. actinomycetemcomitans* specific CD4+ clone would only accumulate in the gingival tissue of rats which had been infected with this organism (369). Thus, a system for the potential kinetics of T-cell entry into gingival tissues and their retention has been described for the first time.

Since the indications are that the periodontitis lesion in the susceptible subject is B-cell dominated, the immunoglobulins being produced by plasma cells at the site, their subclass distribution and relationship to immunoglobulins detected in the GCF are of relevance to local and systemic immunity. In the present study, therefore, an investigation was carried out into the distribution of IgG1-4, IgA1-2 and light chain κ and λ producing plasma cells in the gingivae of periodontitis subjects, and their relationship to actual levels of immunoglobulin detected in the GCF.

1.7 Overall aims of the study

The aims of this study fall under five broad headings. The first was to follow up previous work in this laboratory which had indicated that local disease status is related to levels of specific immunoglobulin. A previous study had shown that gingivitis and periodontitis sites can be distinguished in that the latter have lower antibody levels to *P. gingivalis* in the GCF than the former (338), perhaps indicating local degradation by oral pathogens (145). In the present study, the experimental design was modified to allow matching of gingivitis and periodontitis sites within the same patient, generating the potential for a more powerful statistical analysis.

Reports demonstrating differences in specific humoral immunity with ageing (212-213) prompted an experimental gingivitis study on periodontally healthy young and old subjects, to investigate possible differences in local immune and inflammatory parameters. Since chronic periodontal disease is primarily a disease of ageing, any immune deficiencies contributing to periodontal disease may be related to the ageing process itself.

A previous report from this laboratory had suggested that there may be differences in immune and inflammatory processes at matched oral implant and natural tooth sites in the same patient (341). Part of this study, therefore, was to follow up these investigations.

Checkerboard immunoblotting may have the potential to allow multiple assessments of local antibody responses to oral pathogens from a GCF sample (370). Therefore, a pilot study of the feasibility of this technique was included as part of this study.

The second aim was to investigate local production of immunoglobulins by plasma cells in the gingiva. Therefore, an investigation was conducted into the ratios of κ and λ light-chain producing plasma cells and their possible relevance to periodontal disease. Another study dealt with production of IgG and IgA subclasses by plasma cells in the gingiva and related these to levels of the immunoglobulins in the GCF. Degradation of IgA1 was also assessed, to determine the significance of bacterial digestion of host immunoglobulin in the periodontal pocket.

Previous work in this laboratory has demonstrated the significance of specific serum antibody avidity to

periodontal diagnosis and prognosis (330,347). Therefore, the third aim of this study was to investigate the impact of antibody avidity on the outcome of periodontal treatment. A preliminary study was set up to assess these effects; also attempting to relate microbiological parameters. A larger study was then conducted in which patients were assigned to seropositivity groups according to the definition of Chen et al (273) at baseline. They were then followed longitudinally to assess differences in treatment outcome. Since earlier studies had suggested the particular relevance of *P. gingivalis* antigens such as the ARG-1 protease, a separate study was carried out into the prognostic relevance of antibody avidity to this antigen.

A fourth aim of this study was to look at the wider significance of immunological parameters in disease pathology. Periodontal disease can in some ways be seen as a model immune-inflammatory process. By the same token, the immunology of pregnancy has some unique characteristics making it worthy of study. The relevance of serum levels of IgG subclasses to pregnancy outcome was assessed. In addition, levels of an auto-antibody were assessed as a possible outcome marker.

The fifth aim of the study, related to the above, was to determine if bacterial fragments can enter the human bloodstream. If so, and these fragments can then enter the foetal circulation via the placenta, then this *in utero* exposure may explain the very weak antibody avidity response to oral pathogens in humans (190). Tolerisation may occur, either by a mechanism akin to oral tolerance or the action of an IgG blocking factor as seems to occur with paternal antigens in pregnancy.

Thus the overall aim of this project is to increase understanding of the immunological aetiology of periodontal disease at local and systemic, cellular and molecular levels; and to put these findings into the wider context of immune-inflammatory processes.

Chapter 2

Materials and Methods

2. Methods

2.1 Local antibody studies

2.1.1 Specific antibody levels in GCF at healthy, gingivitis and periodontitis sites within the same patient

Clinical Criteria

Forty patients, (26 F; 14 M; mean age 43.1 (SD=10.9)) attending the Periodontal Clinic of Glasgow Dental Hospital participated in this study. These individuals had no history of systemic disease and had not received antibiotics for the past three months. Three sites (healthy, gingivitis and periodontitis) from each patient comprised the study sites. Using the modified gingival index (MGI) (371) and a pressure sensitive probe with a constant force of 25g, sites with gingival scoring of 0 or 1 and pocket depth (PD) less than 2mm were categorised as healthy. MGI between 2 and 4 and PD<3mm were the criteria for gingivitis sites, and sites with MGI of between 0 and 2 and PD>4mm comprised the periodontitis sites (Table 1.).

Although difficulties were experienced in finding the three types of sites (healthy, gingivitis and periodontitis) within the same patient, there were considerable advantages with this protocol since the subject variability could be excluded by pairing three sites to be compared in the statistical analysis. The following criteria for site selection were adopted: mesial sites were preferred to distal sites since they were more accessible; upper molar palatal sites were preferred to buccal sites; and on the lower teeth buccal/labial sites were preferred to lingual sites, since these sites were less prone to any possibility of saliva contamination of the GCF sample.

Gingival Crevicular Fluid (GCF) Sampling and Processing

GCF samples were collected using Whatman grade 4 paper strips (2x13 mm) (372). The site being sampled was gently air-dried and any supragingival plaque was removed. The area was carefully isolated to prevent samples being contaminated by saliva. The paper strip was inserted into

the crevice until mild resistance was felt, and left for 30 seconds. Care was exercised in order to avoid mechanical injury of the tissues. After GCF collection, the paper strip was transferred to the chairside-located Periotron 6000 (Harco Electronics, Winnipeg, Canada) for the quantification of the fluid volume. The jaws of the Periotron were wiped with pure methanol between each reading. The strips were then placed in individual sterile tubes and stored at -30°C until further processing.

Subsequently, the strips were eluted into 500μl of phosphate-buffered saline containing 0.05% Tween 20 (PBST) at room temperature using a rotary mixer. After removal of a small volume (20μl) for albumin analysis, the remainder was diluted with an equal volume of PBST containing 0.2% bovine serum albumin (BSA), giving a final concentration of 0.1% BSA. This procedure allowed the albumin samples to be eluted into an albumin-free buffer, while also allowing the antibody samples to be eluted into the assay buffer with the appropriate blocking agents (0.05% Tween 20 and 0.1% BSA). The strips were discarded and the eluate aliquoted and stored at -30°C.

Calibration of the Periotron 6000

In order to transform the Periotron digital readings for each paper strip into volumes and also assess the accuracy of the instrument, known volumes of distilled water were delivered to Whatman grade 4 paper strips with a Hamilton microsyringe in a range of volumes (0.05-1μl). Each measurement was performed 3 times and the mean value for each volume was used in a linear regression analysis from which the slope and intercept were used to determine the volumes of GCF collected.

Anti-bacterial antibody ELISA

Specific antibody titres were measured by ELISA based on the method of Ebersole et al (373,374), using formalinised whole cells at an absorbance (OD_{600}) which had previously been determined as optimum to coat microtitre plates. The bacterial strains used was *P. gingivalis* NCTC 11834. *P. gingivalis* was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2). *P. gingivalis* was harvested after 5 days into phosphate-buffered saline (pH 7.4), with 1mM Na_2EDTA,

washed by centrifugation, and fixed for 1 hour in 10% formal saline. The cells were then washed twice in PBS and once in 0.1M Na carbonate-bicarbonate buffer containing 0.02% NaN_3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at $4^{o}C$ until use.

Immulon 1 plates (Dynatech) were employed because of their low protein-binding characteristics. After coating, the plates were treated with PBS containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20 and 5% skimmed milk to remove background binding. GCF diluted in this buffer, to a concentration within the range of the calibration graph, were then added for 2 hours at $37^{o}C$, and the plates were subsequently incubated with biotin-anti-human IgG (150ng/ml), IgA (220ng/ml) or IgM (170ng/ml) (Sigma) and thereafter with 1mg/ml extravidin-peroxidase (Sigma).

Reaction was visualised using o-phenylenediamine substrate and stopped with 1M H_2SO_4. Optical densities were read at 490nm. Samples were assayed in triplicate, correction was made for non-specific binding and results were read from a reference line derived from serial dilutions of a reference positive control serum. Results were expressed as ELISA units (EU) (375).

Quantification of albumin

Albumin was assayed in GCF by sandwich ELISA based on the technique described by Hetherington *et al.* (376) and modified by Adonogianaki *et al.* (377). In brief, the 96-well polystyrene microplate (Immulon 4, Dynatech Laboratories, Billingshurst, Sussex) was coated with the first antibody, a goat antiserum specific for albumin (1/6000 dilution in carbonate/bi-carbonate pH 9.6 coating buffer). The eluate of the sample was then added and any antigen present was captured by the immobilised antibody. This was followed by incubation with the second specific antiserum, developed in rabbit at 1:4000 dilution in incubation buffer (PBS containing 0.05% Tween 20). Both of these antisera were obtained from Sigma. Finally, the horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) (Jackson Laboratories) was added (1:4000 dilution in incubation buffer). Visualisation was achieved by incubation with the substrate and stopping the reaction with H_2SO_4. The plate was read at 490nm. Plates included serial two-fold dilutions of purified antigen for the construction of a standard curve. The working range for the albumin ELISA was 1.95-125 ng/ml.

ELISA buffers

The buffers employed in ELISA assays throughout this entire study were as follows except where specifically mentioned in later sections:-

1. Coating buffer: 1.59g Na_2CO_3, 2.93g $NaHCO_3$ in 800ml distilled H_2O, dissolve, pH to 9.6 at just under 1L, add 0.2g NaN_3, make up to 1L in volumetric flask. Store in sterilised bottles at 4C for 1 week.

2. Wash buffer (x10 conc.): 80g NaCl, 14.4g $Na_2HPO_4.2H_2O$, 2g KH_2PO_4, 2g KCl, 5g Tween 20. Dissolve in 800 ml of distilled H_2O, make up to 1L and store at RT diluting 1/10 immediately pre-use.

3. Incubation buffer: 1/10 of the quantities added to above (2) made up to 1L with the addition of 1g of BSA. This should be layered on the surface without mixing. Store at 4°C for maximum of 1 week.

4. Substrate buffer: 0.35g Na_2HPO_4, 0.2375g citric acid. Dissolve, make up to 25ml in measuring cylinder and add 1 tablet (10mg) of oPD and 10μl 30% hydrogen peroxide, mixing well, immediately before use.

Statistical analysis

The non-parametric Wilcoxon signed rank test was used to perform paired comparisons of the non-normally distributed titre data for healthy, gingivitis and periodontitis sites within the same individual.

2.1.2 Effects of ageing on local humoral immunity and inflammatory responses in an experimental gingivitis study

Subjects and study design

Five young individuals, 20-25 years of age and 5 older individuals, 65-80 years of age volunteered to participate in the study. The subjects in the 20-25 year age category were all dental students, while the subjects in the 65-80 year age group were patients of the Comprehensive Dental Care Unit, Community Dental Clinic, Göteborg, Sweden. The study protocol was approved by the Human Subjects Review Committee of Göteborg University.

All subjects received professional tooth cleaning during 4 weeks and had at the end of this preparatory period excellent oral hygiene, clinically healthy gingivae and no deepened periodontal pockets. A baseline examination which included assessment of plaque and gingivitis was performed on Day 0. The examination was restricted to the *experimental sites*, namely the mesio-

palatal, palatal, and disto-palatal surfaces of the teeth present in the 15....25 tooth region. 4 sites were selected for gingival fluid assessment (*fluid sites*); in addition, as part of a larger study in Göteborg, 3 sites were selected for biopsy (*biopsy sites*), and a further 3 sites for microbial sampling (*microbial sites*) within the experimental sites. The biopsy and microbial samples were taken as part of a larger study and, therefore, no data on these aspects appears here.

Gingival crevicular fluid sampling

As in section 2.1.1.

Quantification of α2-M and LF

α2-M, and LF in eluates of GCF were assessed using sandwich enzyme linked immunosorbent assays (ELISA).

These are based on the technique described by Hetherington et al. (376) and modified by Adonogianaki et al (377). In brief, the 96-well polystyrene microplate (Immulon 4, Dynatech Laboratories, Billinghurst, Sussex)

was coated with the first antibody, a goat antiserum specific to the antigen to be quantified (1:6000 dilution in carbonate/bi-carbonate buffer). The eluate of the sample was then added and any antigen present was captured by the immobilised antibody. This was followed by incubation with the second specific antiserum, developed in rabbit (1:4000 dilution in incubation buffer). Finally, the horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was added (1:4000 dilution in incubation buffer). Visualisation was achieved by incubation with the substrate and stopping the reaction with H_2SO_4. The plate was read at 490nm, and optical densities (ODs) obtained using the Dynatech Minireader II (Dynatech Laboratories, Alexandria, VA). Plates included serial two-fold dilutions of purified antigen for the construction of a standard curve. Only the central wells were used when running standards or samples (in triplicate). The peripheral wells were used for assaying the controls. All controls were run in duplicate except for the zero-antigen control which was run in quadruplicate. The working range of the standard for each of the above assays was determined prior to assaying and GCF sample eluates were diluted further in IB in order to

achieve an optimal final dilution for each assay. Purified α2-M was obtained from SIGMA (SIGMA Chemical Company Ltd., Poole, Dorset) whereas LF was purchased from Calbiochem (Novabiochem Ltd., Nottingham). Goat and rabbit anti-α2-M were also obtained from SIGMA. Goat anti-LF as well as rabbit anti-LF were obtained from Nordic Immunological Laboratories (Maidenhead, Berkshire). The horseradish peroxidase (HRP) conjugated anti-rabbit IgG (goat) was purchased from ICN Immunobiologicals (Lisle, IL, USA). Of the above antisera the rabbit antiserum to α2-M and the HRP conjugate were fractionated.

Quantification of albumin

This was in section 2.1.1.

ELISA assays for total IgG1, IgG2, IgG3 and IgG4 subclass levels in GCF.

These assays were broadly based on the sandwich ELISA system previously developed (85). However, these were substantially modified to increase sensitivity and thus detectability in GCF.

Microtitre plates (Immulon 4, Dynatech Laboratories, Billingshurst, UK) were coated with monoclonal anti-human IgG1, IgG2, IgG3 or IgG4 (Sigma, Poole, Dorset, UK). These monoclonal antibodies have been shown to be completely specific for their respective subclasses (378). This was followed by incubation with a standard human serum, the IgG1-4 subclass concentration of which had been determined by nephelometry (Department of Clinical Immunology, Western Infirmary, Glasgow). This allowed reference lines for IgG1-4 concentrations to be constructed.

The plate was then incubated with biotin-anti-human IgG (Sigma) followed by an incubation with Extravidin-peroxidase (Sigma - TM) thus achieving amplification of signal strength. Reaction was visualised with tetramethylbenzidine (TMB) substrate (KPL, Gaitersburg, Maryland, USA), stopped with 2.5% sodium fluoride and plates were read at 630nm with 490nm reference on a MR5000 Multiskan (Dynatech Laboratories). GCF levels were calculated by reference to linear regression analysis of the standard serum line.

Statistical analysis

Protein levels were not normally-distributed and therefore comparisons between the young and old groups was conducted using the Mann-Whitney test.

2.1.3 Comparison of the local immune response in osseointegrated implants and natural teeth.

Patients and sites

Twenty partially edentulous subjects (8 males, 12 females; age range:42 to 86 years) at Bern Dental School, Switzerland who had no history of systemic conditions which could influence the course of periodontal disease and had not been on antibiotics for the previous two months, were selected to participate in this study. The participants had at least one osseointegrated implant, which had been in function for a minimum of six months (average: 9 months; range: 6 months to 12 months). The sites had to conform to the following criteria:-

1. No bleeding on probing.
2. probing pocket depths < 4mm.
3. absence of visible plaque (PI = 0).
4. Clinical and radiographic signs of osseointegration at implant sites.

Processing of gingival crevicular fluid (GCF)

As in section 2.1.1.

GCF and PISF sampling

As in section 2.1.1.

Anti-bacterial antibody ELISA

This was as in section 2.1.1.

Quantification of albumin

This was as in section 2.1.1.

Relative coefficient of excretion (RCE) calculation

Total IgG, IgM, IgA and albumin was assayed in GCF or PISF and serum from the same patient and the RCE value as used by Out *et al* (379) was calculated. The formula applied was as follows:-

$$RCE = \frac{\dfrac{Ig\ (GCF\ or\ PISF)}{Ig\ (serum)}}{\dfrac{albumin\ (GCF\ or\ PISF)}{albumin\ (serum)}}$$

Since albumin is only systemically derived, RCE values >1 indicate a preponderance of locally produced immunoglobulin and thus this ratio gives a value directly proportional to the extent of local immunoglobulin production.

Statistical Analysis

Since values for titres and RCE ratios were non-normally distributed, the Wilcoxon signed rank test was used to determine whether or not the matched paired values were significantly different between natural teeth and implants. Multiple regression analysis was used for correlations.

2.1.4 Checkerboard immunoblotting of gingival crevicular fluid

Clinical criteria

Between four and six sites were sampled in each of 43 RPP patients. A range of pocket depths was sampled in each patient i.e. <4mm and ≥4mm.

Collection of GCF

As in section 2.1.1.

Immunoblotting technique

This was based on the method of Kazemi and Finkelstein (370). The immunoslotting and immunoblotting equipment was purchased from Immunetics (Cambridge, MA, USA). Sonicated bacterial antigen was applied to nitrocellulose C-extra membranes (Amersham International, Little Chalfont, Bucks.) under vacuum in horizontal slots. The membranes were then blocked

overnight in PBS containing 0.2% Tween 20 and 2.5% non-fat dried milk (Marvel). GCF was diluted in the blocking buffer and blotted onto the membrane vertically. After washing with PBS-Tween, the vertical channels were further incubated with a monoclonal antibody to human IgG conjugated to alkaline phosphatase (Sigma). The development substrate was NBT/BCIP (see methodology in section 2.2.).

2.2 *In situ* hybridisation studies on plasma cells

2.2.1 Study of κ/λ expressing plasma cells

Seventeen gingival tissue samples were obtained during periodontal surgery under local anaesthesia following the initial hygiene phase treatment of these sites, from 8 patients with adult periodontitis attending the Periodontology Unit, Glasgow Dental Hospital & School. In all cases pocket depth were >5 mm with radiographic evidence of bone loss. Tissues were immediately immersed in 10% neutral buffered formalin at room temperature. The tissues were blocked in a plane parallel to the long axis of the teeth and oriented so that pocket epithelium, oral epithelium and connective tissue were present in the same section. They were embedded in paraffin wax and 5 μm sections were made onto silane (Sigma Chemical Co., St. Louis, MO) coated glass slides.

Immunoglobulin light chain mRNA-expressing cells were detected by *in situ* hybridisation as described in the protocol of DAKO κ/λ mRNA ISH kit (Code No. K045) and ISH Detection Kit (Code No. K046) with minor modifications.

The buffers used in the *in situ* hybridisation procedure were as detailed in section 2.2.2. All solutions were prepared with 0.1% diethyl pyrocarbonate (Sigma) treated distilled water. Briefly, three distinct areas of each biopsy were studied and consecutive serial sections from each area were deparaffinised in xylene, hydrated through descending ethanol, and washed in distilled water, then immersed in 0.2 M HCl at room temperature for 20 minutes to remove basic proteins. Then the sections were digested with proteinase K (0-50 μg/ml, Sigma) in 0.1 M Tris-HCl, pH 8.0, containing 50 mM EDTA for 30 minutes at 37°C. After immersing in 0.2% glycine/distilled water for 30 seconds, they were washed twice for 5 minutes in distilled water. Post-fixation was performed by incubation in 0.4% paraformaldehyde for 20 minutes at 4°C, and then washed twice for 5 minutes in distilled water. After drying with ethanol, the sections were hybridised with 20μl volumes of the mixtures containing fluorescein isothiocyanate (FITC)-conjugated κ or λ oligonucleotide probes and hybridisation buffer (1:1). They were then covered with dimethyldichlorosilane-(BDH, UK)-coated coverslips, and incubated overnight at 37°C in a humid chamber. The slides were rinsed in x2 standard sodium citrate for 5

minutes, sequentially, in Tris-buffered saline (TBS; 0.05M Tris/HCl, 0.15M NaCl, pH 7.6)/0.1% Triton X (Sigma) for 10 minutes, and then immersed in TBS. The slides were incubated with diluted (1:80) rabbit F(ab) anti-FITC/alkaline phosphatase conjugated secondary antibody for 30 minutes in room temperature. After immersing in TBS for 5 minutes, the slides were rinsed in detection buffer (1.21% Tris base, 0.58% NaCl, 1.0% $MgCl_2$ pH 9.5) for 5 minutes and developed with enzyme substrates (Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate) with levamisole for 30-45 minutes, and then washed in distilled water three times. Finally, the sections were counterstained with 1% Neutral Red and mounted in aqueous mounting medium (DAKO).

κ or λ positive cells were evaluated separately in serial sections. Five distinct microscopic fields which showed the strongest cell infiltration, from each serial section of all seventeen biopsies, were chosen for counting using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 mm^2). The statistical significance between κ to λ chain mRNA positive cells was analysed by Wilcoxon's matched-pairs signed-ranks test. The percentages of κ and

λ immunoglobulin light chain positive plasma cells and the κ to λ ratio were also calculated. In addition, the mean percentage of patient plasma cells which were κ light chain positive and the patient standard deviation were also calculated.

2.2.2 Study of IgG1-4 and IgA1-2 expressing plasma cells including comparison with immunoglobulin levels in GCF

Tissues

Twenty-four gingival tissue samples were obtained during periodontal surgery, after GCF sampling was performed, from 8 patients with adult periodontitis attending the Periodontology Unit, Glasgow Dental Hospital for periodontal therapy. The pocket depths of the sampled sites were greater than 5 mm with radiographic evidence of bone loss. The tissues were cut from buccal to lingual gingiva in a plane parallel to the long axis of the teeth and oriented so that pocket epithelium, oral epithelium and connective tissue were present in the same section. The tissue sections were transferred to 10% neutral buffered formalin at room temperature and subsequently embedded in paraffin wax after which 5 μm serial sections were made onto silane-coated glass slides.

Preparation of oligonucleotide probes

Probes were normally supplied on columns by the Department of Veterinary Pathology, University of Glasgow. The probe was eluted from the column with 40 % ammonia, incubated at 55°C overnight and then precipitated with 3M sodium acetate in 2% ethanol and 0.4% ammonia at -70°C for 30min. The solution was then centrifuged at 13,000 rpm for 10-15 min in a microcentrifuge and the supernatant removed. The pellet was washed with 1 ml 70 % ethanol followed by a further spin at 13,000 rpm for 5 min. The pellet was then air-dried and resuspended in TE buffer, and quantified by spectrophotometry (260 nm). The probe was then stored at - 20°C until required.

Probe labelling

This essentially used the Boehringer kit method (cat. no. 1093088) with some reagents being obtained elsewhere. The first stage was the tailing reaction in

which digoxigenin was attached to the probe, and the following mixture was used:-

5x stock tailing buffer (Gibco)	4 μl
Dig-11 dUTP (1 nmol /ml) or Bio-16dUTP	2 μl
Probe (oligonucleotide adjusted to 1-2mg/ml)	2 μl
TdT enzyme (15 U/ml)	2 μl
DEPC/dH_2O	10 μl

This was then mixed and centrifuged briefly in a microcentrifuge (13000 RPM), then incubated overnight at 37°C.

The second stage involved isolation of the probe by separation on a Sephadex G25 NAP™ - 5 column. The running buffer was x0.1 SSC + 0.1% SDS in DEPC/dist.H_2O. 10 x1ml fractions were collected and the fractions containing labelled probe were identified by dotblotting. 1μl samples were dotblotted onto a nitrocellulose filter and baked in a UV-cross linker at 1200W for 30s. Immunological detection was achieved as described below using digoxigenin-3′-labelled control oligonucleotide as a standard conrtol.

Oligoprobes employed

Synthetic digoxigenin-labelled oligonucleotide anti-sense human IgG1, IgG2, IgG3 and IgG4 oligonucleotide probes were constructed (GCA CGG TGG GCA TGT GTG AGT TTT GTC ACA AGA TTT GGG CTC; TGG GCA CGG TGG GCA CTC GAC ACA ACA TTT GCG CTC AAC TGT; A AGA TTT GGG CTC TGG GCA CCG TGG GCA TGT GTG AGT TGT; AA CTC AGG TGC TGG GCA TGA TGG GGG ACC ATA, respectively) as were synthetic digoxigenin-labelled oligonucleotide anti-sense human IgA1 and IgA2 gene (CC GGA TTT TGA GAG GGT GGC GGT TAG CGG GGT CTT GGA CTC GGG GTA GGC; CC GGA TTT TGT GAT GTT GGC GGT TAG TGG GGT CTT CAA CTC GGG GTG GGC) (380-384). The absence of cross-hybridization of the two probes was confirmed by Islam *et al* (380). For double staining an ABC-alkaline phosphatase kit (Vector AK-5002) was employed to label the IgA probe with biotin. Subsequent reaction with avidin-peroxidase and a phenylenediamine peroxidase substrate produced a brown product. This contrasted with the earlier blue NBT/BCIP product produced by the alkaline-phosphatase antibody to digoxigenin-labelled IgG probe.

Thus, this technique allowed sequential detection of different immunoglobulin mRNA in the same section. The anti-sense probe sequences for IgA and IgG were GTC ACA TCC TGG CTG GGA TTC GTG TAG TGC TTC ACG TGG CAT GTC ACG GAC (381) and GGC TGC CTG GTC AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA (384) respectively.

***In situ* hybridization**

The sections were depariffinised in descending alcohols then washed twice in PBS for 5 min. Proteinase K (5µg/ml, Sigma) digestion was then carried out in 0.1M Tris-HCl, pH 8.0 containing 50mM EDTA for 30 min at 37°C. After immersion in 0.2% glycine distilled water for 30s, they were washed twice for 5 min in PBS. At this stage, pre-treatment with RNAse A type 1A (Sigma) at 200µg/ml in 2x SSc/10mM $MgCl_2$ at 37°C for 1h was used as negative control. Postfixation was by incubation in 4% paraformaldehyde at 4°C for 5 min, followed by two washes in PBS for 5min. The sections were then immersed in prehybridisation buffer (2x standard SSC and 50% formamide) for 2h at 37°C.

After draining, the sections were hybridized with 20 µl volumes of the mixtures containing digoxigenin-labelled probes at a concentration of 0.16 - 0.65 ng/µl in the hybridization buffer. The slides were covered with

dimethyldichlorosilane-(BDH, UK)-coated coverslips that were sealed with rubber cement, and incubated at 37°C overnight. The slides were rinsed in x4 SSC, sequentially, in x2 SSC at room temperature twice for 20 min, x0.1 SSC at 37°C twice for 20 min, then washed in x2 SSC for 10 min at room temperature.

Immunological detection

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

Buffers employed

1. DEPC (Diethyl pyrocarbonate)/PBS or double dist. H_2O:-

1 ml DEPC was added per 1 litre of PBS (or dH_2O) then mixed well and allowed to stand overnight before autoclaving.

2. Proteinase K (PK) solution:-

The stock solution was 500 mg/ml in DEPC/dH_2O and was stored in 100 μl aliquots at -20°C.

3. PK dilution buffer:-

0.1 M Tris/HCl, pH 8.0 with 0.05 M EDTA) filter sterilised with a 0.45 μm filter.

4. TE buffer:-

0.01M Tris/HCl, pH 7.5 with 0.01M EDTA filter sterilised with a 0.45 μm filter.

5. Standard sodium citrate (SSC):-

Sodium chloride 0.15 M, Sodium citrate 0.015 M prepared in DEPC/ dH_2O as x20 stock solution and used at x4, x2, x1 and x0.1 concentrations.

6. Detection buffer 1:-

0.25M Tris Hcl, 0.3M NaCl, pH 7.5.

7. Detection buffer 3:-

0.1M Tris/HCl, 0.1M NaCl, 0.05 M $MgCl_2$, pH9.0.

8. Nitro-blue tetrazolium/bromochloro-indolylphosphate (NTB/BCIP):-

Stock NBT soln: 75 mg/ml NBT in 70 % dimethylformamide.

Stock BCIP soln: 50 mg/ml BCIP in 100 % dimethylformamide.

Both solutions were light sensitive and were stored in foil-wrapped glassware at 4°C.

9. Hybridisation buffer:-

This was 50% formamide, 10% dextran Sulphate, 0.5% SDS, 0.3% Triton, in 0.01M Tris, x12.5 Denhardts, x2 SSC, made up to final volume and concentration in DEPC/dH_2O.

10. Denhardts x100 stock solution:-

20mg/ml BSA, 20mg/ml Ficoll, 20mg/ml in DEPC/dH_2O.

The specific functions of reagents

The more important specific functions of reagents are summarised below:-

BSA & salmon sperm DNA is used to block non-specific binding of the probes.

DEPC Inhibits the effect of RNAses. This is an important aspect of inhibition of endogenous and exogenous RNAses allied with precautions such as autoclaved glassware, dust-free storage and wearing of gloves.

Formamide destroys hydrogen bonding, making it easier for probes to anneal with mRNA in tissue sections at low temperatures.

Proteinase K is an enzyme used to digest the tissue in order to allow optimum access of oligoprobe. The optimisation of protienase K concentration used for digestion is the most crucial determinant of the quality of staining.

Glycine is a proteinase K inhibitor used to inhibit further digestion.

Levamisole is an endogenous alkaline phosphatase (AP) inhibitor used to ensure that only exogenous antibody-bound AP is detected.

Paraformaldehyde is employed in post-fixation of the tissue.

Prehybridisation buffer is used as a blocking agent before the hybridisation step.

Silane acts as a "glue" allowing sections to adhere more efficiently to glass slides.

SSC is used in the stringency washing of sections i.e. they are subjected to decreasing sodium concentration and increasing temperature to detach non-specific binding of probes.

Controls for *in situ* hybridization

As a negative control, sections were incubated with hybridization buffer only or sense probes. To determine the specificity of probe bound to tissues RNAs, sections were digested with RNAse prior to hybridization. Other specificity controls were carried out by hybridizing with the labelled probe together with the unlabelled probe simultaneously. The concentration of the former was as above, but the latter was in 10-fold excess. For example, labelled IgA1 probe with 10-fold non-labelled IgA2 were used to check the specificity of the IgA1 probe and vice versa. In addition, double-target *in situ* hybridization methodology has been developed in this laboratory using

digoxigenin- and biotin-labelled IgG and IgA subclasses probes and it has been confirmed here that there were no cross reactions between the probes (manuscript submitted).

Cell counts

As previously described, twenty-four gingival biopsy samples were obtained from 8 patients. Briefly, five distinct microscopic fields which showed the strongest cell infiltration, from each serial section of all seventeen biopsies, were chosen for counting using a meshwork eyepiece (0.172 mm x 0.172 mm; 0.0296 mm^2). IgG and IgA subclass specific mRNA-expressing cells were enumerated in each serial section. The percentage of each IgG and IgA subclass as compared to the total IgG or IgA positive cells were also calculated (e.g. (IgG1/(IgG1 + IgG2 + IgG3 + IgG4)) x 100 %). The percentage of IgA1 subclass positive cells were also calculated (IgA1/(IgA1 + IgA2)) x 100 %.

Gingival crevicular fluid sampling

As in section 2.1.1.

Determination of IgG subclass proteins in GCF

As in section 2.1.2.

Determination of IgA subclass protein and IgA1 fragment levels in GCF

In the IgA1 assay, microtitre plates (Immulon 4, Dynatech Laboratories) were coated with anti-human IgA (Scottish Antibody Production Unit, Carluke, UK). This was followed by incubation with a standard human serum the IgA concentration of which had been determined by nephelometry (Department of Clinical Immunology, Western Infirmary, Glasgow). By jacalin absorption the relative concentrations of IgA1 and IgA2 in this serum were determined (385). This allowed a reference line for IgA1 concentration to be constructed. The plate was then incubated with monoclonal biotinylated anti-human IgA1 (Sigma, Poole, Dorset, UK) followed by an incubation with Extravidin-peroxidase (Sigma - TM) thus achieving amplification of signal strength. Reaction was visualized with tetramethylbenzidine (TMB) substrate (KPL, Gaitersburg, Maryland, USA), stopped with 2.5% sodium fluoride and plates were read at 630 nm with 490 nm

reference on a MR5000 Multiscan (Dynatech Laboratories). GCF levels were calculated by reference to linear regression analysis of the standard serum line. The IgA2 assay was similar except for the coating and second antibodies employed. Plates were coated with monoclonal anti-human IgA2 (Boehringer Mannheim). The second antibody was biotin-anti-human IgA (Sigma). The methodology of the IgA1 Fab fragment assay was based on that of Frandsen *et al.* (146). Briefly, plates were coated with a monoclonal antibody to the IgA1 neo-epitope (kindly provided by Dr EVG Frandsen), followed by incubation with GCF and development with biotin-anti-human IgA1 monoclonal antibody (Sigma).

Statistical analysis

Wilcoxon's matched-pairs signed-ranks test was used to determine differences between subclass ratios of IgG and IgA mRNA-expressing cells and these proteins, and between the amount of IgA1 Fab fragments and that of intact IgA1.

The correlation between the data of the amount of intact IgG and IgA subclass protein and the numbers of these subclass specific mRNA-expressing cells per section was estimated by Spearman's rank correlation coefficient.

2.3 Studies of systemic antibody avidity

2.3.1 Preliminary longitudinal study in patients with refractory periodontitis

Patient selection and treatment regimen

Ten adult Caucasian patients (three males, seven female mean age 42±8 yr, range 34-57 yr) were recruited from a recall pool of 600 in a specialist periodontal practice in Northern Iceland. Eight patients were regular smokers. All gave informed consent to participate in the study and had no systemic disorders. They had not received antibiotics in the six months prior the baseline investigation. All patients had 20 or more teeth and had lost more than 50 percent of supporting alveolar bone around at least two teeth in each quadrant. In addition, all patients had successfully completed periodontal treatment, including surgery, at least two years before being enrolled in the study and had received supportive hygiene therapy three times a year thereafter until the study commenced. Sites for

inclusion in this study were selected in groups of three: (a) one treated by open debridement; (b) one by root planing; and (c) the third scaled supragingivally, which served as a control. The selection of sites and protocol for the surgical and non-surgical treatment phase of the study has been reported elsewhere (386).

Antibiotic regimen

Between weeks 53 and 55, all patients had were treated with amoxycillin and metronidazole both at doses of 250 mg three times daily for 10 days.

Control subjects

Ten control subjects recruited from staff of the Dental Faculty in the University of Iceland were matched for age and sex with the patients. It also proved possible to match three controls with patients according to smoking pattern and a further two controls smoked occasionally. The two patients that did not smoke were matched with non-smoking controls. All gave their informed consent to the investigation. Samples of

subgingival plaque were removed from four quadrants using paper points and sent to the laboratory for culture. Control subjects were selected on the basis that they did not carry *A.actinomycetemcomotans* or *P. gingivalis* in these samples. One serum sample was then collected from each control and stored frozen as described for the serum samples from patients.

Microbiological investigation

Microbiological sampling of the gingival pockets under investigation was performed at baseline (week 0), two weeks after periodontal therapy (week 4) and then at weeks 6, 26 and 52. The patients received antibiotics for two weeks (weeks 53-55) and microbiological sampling was repeated at week 75. Subgingival paper point samples were collected from all lesions after the removal of supragingival plaque with sterile curettes and cotton-wool pellets. Three paper points were placed into each pocket, removed after 10 seconds and placed immediately into 1 ml of VMG II transport medium (387). Air in the headspace of the sample container was displaced with a burst of CO_2 and the specimen sent to

the laboratory for analysis. After vortexing the sample,100μl aliquots were inoculated on to tryptic-soy agar containing bacitracin and vancomycin (TSBV) (388) and fastidious anaerobe agar (Lab M, Bury, Lancs, U.K.) supplemented with 10 percent horse blood. TSBV plates were incubated in candle jars and examined after two and seven days for the presence of *Aa* which was identified on the basis of colony morphology on this selective medium and a positive catalase reaction. Blood-agar plates were incubated anaerobically for 14 days using the Gas Pak system (Oxoid, Basingstoke, U.K.). Colonies resembling *Pg* in appearance were further identified by ultraviolet light fluorescence (389), MUG test (390) and haemagglutination (391).

Immunological investigations

Serum was collected from all subjects at baseline and again at week 75. All serum was stored frozen at -80°C until analysis could be carried out simultaneously on both test and control samples. Antibody titres and antibody avidities were determined in the Dental School

of the University of Glasgow and the specimens were transferred frozen between laboratories.

Determination of antibody titre

As in section 2.1.1.

Dissociation Assay

The dissociation assay to determine antibody avidity was performed as follows:- After incubation with serum as described above, the wells were treated with increasing concentrations of ammonium thiocyanate (0.2-8.0M). The concentration of thiocyanate required to dissociate 50% of bound antibody was determined by linear regression analysis. This was termed the ID50 and provides a measure of relative avidity as previously reported (392,393).

Statistical analysis

Wilcoxon's Signed Rank Test was employed for the non-parametric testing of paired data (394) (Minitab). The median differences in titres and in avidities of serum

antibodies IgG, IgM and IgA to *Pg* and *Aa* were computed: (i) for the patients' sera at baseline and after the course of periodontal and antibiotic therapy; (ii) for the pre-treatment patients sera group and controls; and (iii) for the post-treatment patients' sera and controls. In all statistical tests a probability of $p<0.05$ was considered statistically significant. Despite the high variability of the data observed in patients and in controls, statistics were computed with all the data due to the small number of subjects.

2.3.2 Study of immunological effects in the course of periodontal treatment

Patients

Seventeen subjects (9 males, 8 females, age range: 33-53) suffering from advanced periodontal disease (probing depths of >5.5mm in at least one site per sextant and at least 2 non-adjacent sites per quadrant with probing depth of >4mm) were recruited for this investigation. These subjects had no history of systemic conditions which could influence the course of periodontal disease and had not received antibiotics during the previous three months.

Two non-adjacent sites per quadrant with probing depth >4mm were selected in each subject and followed during the course of this study. Non-adjacent sites were used so as to reduce the possibility of contamination and ensure independence of the GCF and plaque samples. At the baseline appointment, probing depths (PD) and bleeding on probing (BOP) at these sites were assessed using the Florida Probe (395) and blood samples were taken. Blood was allowed to clot, centrifuged at 750g for 15 minutes, and the sera separated, aliquoted and stored at -30°C.

Patients were treated by an experienced periodontist (EA) with oral hygiene instruction and quadrant scaling and root planing under local anaesthesia (HPT).

Subjects were reassessed using conventional probing charts between six and seven weeks after HPT (post-HPT), a blood sample was taken and PD and BOP measurements recorded at the eight preselected sites using the Florida Probe. The duration of HPT ranged between 6 weeks and 7 months, with a mean of 5.5 months. All of the patients were reassessed after three months (± 2 weeks), which was the first of their maintenance visits. A blood sample was also taken during this appointment. Only the eight study sites were used to assess the response to treatment in terms of % sites that have a PD>3.5mm and BOP. It should be noted, however, that the initial assessment of periodontal disease status was made on the basis of a larger number of sites.

Control subjects

These were twenty-three periodontally healthy individuals (age range 20-40; 12 males, 11 females). The immunological data from these individuals was used to define the serostatus of the patients as described by Chen

et al (273), seropositive patients are those with titres > 2X control median.

Bacteria

P. gingivalis NCTC 11834 was grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) and *A. actinomycetemcomitans* ATCC 29523 (serotype a) in CO_2 at 37C on Columbia blood agar. This medium was routinely supplemented in our laboratory with Vitamin K and haemin for the growth of black-pigmenting Gram-negative anaerobes. Otherwise fastidious anaerobe agar should be used. *P. gingivalis* was harvested after 5 days and *A. actinomycetemcomitans* after 24 hours into phosphate-buffered saline, 1mM Na2 EDTA, pH 7.4 (PBSE), washed by centrifugation, and fixed for 1 hour in 10% formal saline. The cells were then washed twice in PBSE and once in 0.1M Na carbonate-bicarbonate buffer containing 0.02% NaN3 at pH 9.6 (coating buffer). Fixed cells were stored in coating buffer at 4°C until use.

Collection of subgingival plaque samples

Subgingival plaque samples were taken with a single stroke using a separate sterile curette for each sample to prevent cross-contamination and collected in tubes containing 0.5 ml of Fastidious Anaerobe Broth (FAB; Lab M, Bury, England). Samples were immediately transported to the laboratory where they were vortex mixed for 30 seconds. Broths were then stored at -70°C until PCR analysis was performed.

Polymerase chain reaction

For PCR analysis, 90µl of vortex mixed subgingival plaque was added to 10µl of 10x lysis buffer (100mM Tris HCl pH 8.0, 10mM ethylenediamine tetraacetic acid, 10% Triton X-100) and boiled for 5 min. 10µl of this lysate was used in each PCR reaction. The primers used for PCR identification of *A.actinomycetemcomitans* targeted the leukotoxin A gene as previously described (396): LKT2 (5'-GGAATTCCTAGGTATTGCGAAACAATTTGATC-3') and LKT3 (5'-GGAATTCCTGAAATTAAGCTGGTAATC-3'), which give an expected amplification product of 262 base pairs. For identification of *P.gingivalis*, the primers used targeted

the fimbrillin gene as previously described (397): FIM1 (5'-ATAATGGAGAACAGCAGGAA-3') and FIM2 (5'-TCTTGCCAACCAGTTCCATTGC-3'), which gave an expected amplification product of 131 base pairs. PCR amplification using primers LKT2/LKT3 was carried out in reaction volume of 100 µl consisting of 10 µl sample lysate and 90 µl of reaction mixture containing 1 x PCR buffer (10 mM Tris-HCl [pH 8.8], 2.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100), 2.5 units of DynaZyme DNA polymerase (Flowgen), 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP) amd 50 pmol of each primer. When the primer pair FIM1/FIM2 was used, PCR reaction conditions were as above except that the reaction mixture contained 1.5 mM $MgCl_2$. Primers were separated from the other components of the reaction mixture by a layer of wax ('hot start' PCR), preventing the reaction from starting until the wax melted upon commencement of PCR cycling and thus improving the specificity and yield of reaction products. PCR cycling was carried out in a Hybaid OmniGene thermal cycler. After an initial denaturation step of 95°C for 5 min, 33 amplification cycles of denaturation at 95°C for 1 min, annealing of primers at 55°C for 1 min and primer extension at 72°C for 1 min were carried out,

followed by a final primer extension step at 72°C for 10 min. Reaction products were stored at -20°C or analysed immediately by gel electrophoresis. 20 μl of each reaction product was fractionated on a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and visualised on a UV transilluminator.

In order to avoid contamination, strict procedures were employed when carrying out PCR. Separate rooms were used for sample preparation, setting up of PCR reactions and analysis of PCR reaction products. Filter tips were used at all stages of the process, except when adding the sample to the reaction mixture when a positive displacement pipette was used. Negative and positive PCR controls were included with each batch of samples being analysed by PCR. The negative control was a standard PCR mixture with the sample being replaced by 10 μl of sterile water; the positive control used was a standard PCR reaction mixture containing 100 ng of genomic DNA from *A. actinomycetemcomitans* or *P. gingivalis* instead of the sample.

ELISA

This was as in section 2.1.1.

Dissociation Assay

This was in section 2.3.1.

Statistical Analysis

Mean values are given for antibody avidities throughout; whereas median values are given for titres because these were not normally distributed.

Student's paired t-tests were used to assess whether differences in antibody avidity were significant between baseline, post-HPT and the first maintenance visit. Because antibody titres were not normally distributed, the non-parametric Wilcoxon signed rank test was used to perform the parallel assessments. When seropositive and seronegative sub-groups were directly compared in terms of treatment outcome, two-sample t-tests were employed.

2.3.3 Avidity of antibody to ARG-1 protease of *P. gingivalis* related to treatment effects

The patient group was as in section 2.1.4. Avidity analysis was as in section 2.3.2. Recombinant ARG-1 protease was kindly provided by Aduse-Opoku et al (316).

2.4 Studies of immunological changes during pregnancy

2.4.1 Immunoglobulin G subclasses during normal pregnancy and recurrent abortion

Subjects

Five groups were studied.

Group 1 comprised 10 healthy non pregnant women of mean age 25±3.5 years.

Group 2 comprised 8 healthy primigravidae (mean age 23.0±5.1 years) who were sampled in the first, second and third trimesters of an on going pregnancy and at 6 weeks post partum.

Group 3 comprised 7 pregnant women of mean age 26.8±3.6 years with a previous history of recurrent miscarriage (RM) (3 or more consecutive miscarriages), but whose pregnancy on this occasion continued to term. The women were sampled in the first, second and third trimesters and at 6 weeks post partum.

Group 4 comprised 10 first trimester pregnant women(mean age 25.9±2.3 years) with a previous history of RM and

whose pregnancy on this occasion again ended in miscarriage later in the first trimester. At the time of sampling outcome was not known.

Group 5 comprised 7 first trimester pregnant women (mean age 24.6±2.9 years) admitted suffering spontaneous abortion(SA).

Blood samples were obtained and stored at -20°C till required.

Measurement of IgG subclasses

This was as in section 2.1.2.

Statistical analysis

Data are presented as mean ± standard deviation. Statistical analysis of results was performed with a Mann Whitney test. $P < 0.05$ was considered significant.

2.4.2 Thyroid peroxidase (TPO) auto-antibody as a pregnancy outcome marker

Patient group

Group 1 comprised 8 RMc subjects i.e. women who were recurrent aborters but on this occasion continued to term. Group 2 comprised 7 RMa subjects i.e. women who were recurrent aborters and on this occasion aborted again.

Method of assessing TPO auto-antibody

This was according to the ELISA kit method of RSR Ltd., Cardiff.

2.5 Use of PCR analysis to detect bacterial fragments in host bloodstream

The patient group and the method of PCR analysis was in section 2.3.2. A pan-specific bacterial probe was used in this study to determine if bacteria enter the bloodstream at all.

Chapter 3

Results

3. Results

3.1 Local antibody studies

3.1.1 Study of local antibody levels at sites with differing clinical status

The clinical parameters from the three categories of site (healthy, gingivitis and periodontitis) are shown in Table 1. These data show that periodontitis sites had lower levels of inflammation, deeper pockets and greater yields of GCF than gingivitis sites. Therefore, if levels of GCF antibody were simply related to volume collected, then higher median levels would be expected in periodontitis sites than in gingivitis sites. Table 2 shows the titres found in each disease category as EU/30s sample and EU/30s sample per unit albumin expressed as mg/ml. The p-values for the Wilcoxon signed rank tests which were used to perform paired comparisons between different site types are given in Tables 3 and 4. Figure 1 shows a typical ELISA assay once developed.

Table 1. Clinical parameters from the three categories of sites in 40 patients.

Site Status	MGI	PD (mm)	GCF (µl)
Healthy	0.17±0.06*	1.41±0.06	0.092±0.02
Gingivitis	3.00±0.07	2.37±0.12	0.230±0.05
Periodontitis	1.70±0.12	5.71±0.14	0.380±0.06

MGI= modified gingival index

PD = pocket depth

GCF= gingival crevicular volume

* = mean ± SEM

(n)= 40 patients

Table 2. Median IgG antibody levels and antibody levels per unit albumin from the three categories of sites.

	Healthy	Gingivitis	Perio
Median EU/30s sample (Q1-Q3)	13 (8-19)	39 (10-76)	16 (8-57)
Median EU/30s sample/unit albumin (mg/ml) (Q1-Q3)	2.4 (1.5-3.4)	7.1 (1.8-13.8)	2.6 (1.0-9.0)

Table 3. p-values for paired comparisons of EU/30s sample data using Wilcoxon signed rank test.

	Healthy	Gingivitis	Perio
Healthy	-	<0.001	0.006
Gingivitis	-	-	0.033

Table 4. p-values for paired comparisons of EU/30s/unit albumin (mg/ml) sample data using Wilcoxon signed rank test.

	Healthy	Gingivitis	Perio
Healthy	-	<0.001	0.006
Gingivitis	-	-	0.02

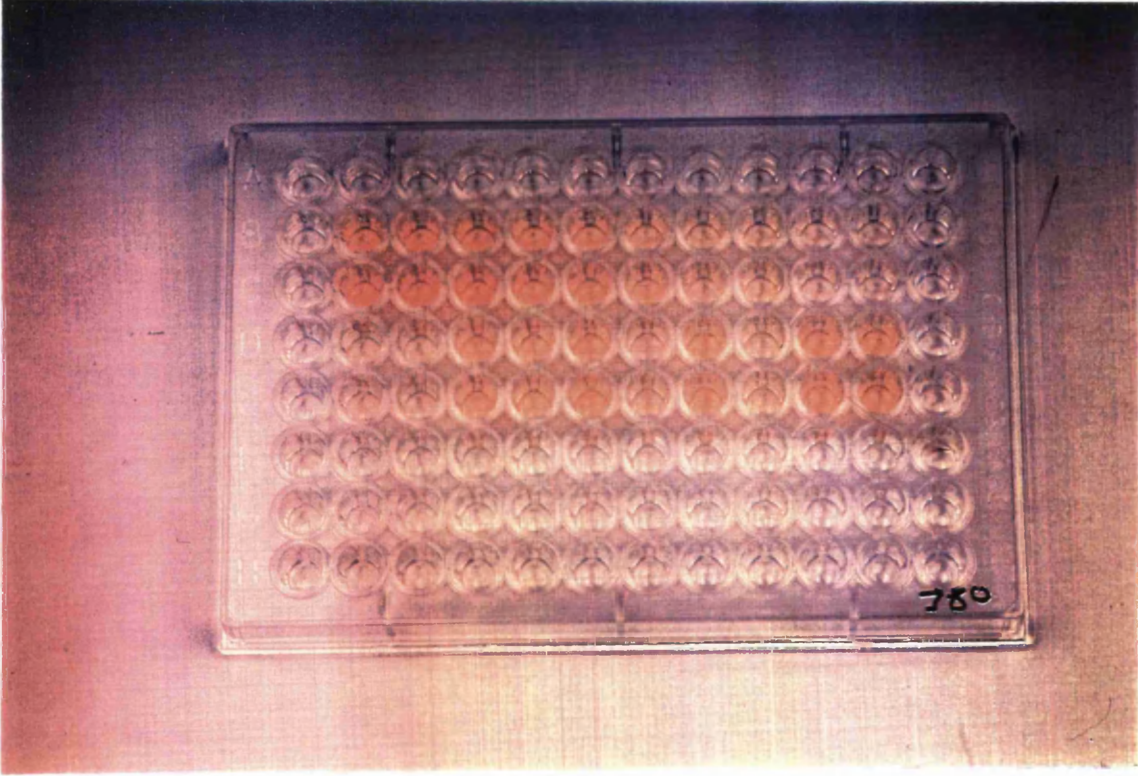


Figure 1: Illustration of typical ELISA plate.

3.1.2 Effects of ageing on local humoral immunity and inflammatory responses

The median concentrations of IgG1-4 for young and old individuals are given in Table 5. These show tendencies towards IgG1 and IgG4 concentrations being higher in GCF in young subjects and IgG3 being lower than in old subjects. All of these differences were bordering on statistical significance at $p<0.05$. Table 6 displays these levels expressed as a ratio to albumin, thus giving a measure of local synthesis. Higher ratios indicate higher levels of local input. Thus there appears to be more local production of IgG3 and less of IgG1 in old than in young subjects. The levels of the other proteins are given in Table 7, suggesting that lactoferrin concentrations are lower in GCF in old individuals than in young individuals. Therefore, PMN Infiltration may be reduced in older subjects. The time-progression points for all of the proteins measured during the course of experimental gingivitis are shown in Table 8. Generally, these show differences in response between young and old individuals in that peaks

Table 5: Median IgG1-4 concentrations (interquartile range in parentheses) for young and old individuals. p-value is given for comparison of two groups by Mann-Whitney test.

	mg/ml		μg/ml	
	IgG1	IgG2	IgG3	IgG4
young	9.9	1.1	60	540
	(4.9-22.4)	(0.5-3.6)	(20-150)	(170-1300)
old	5.5	1.1	120	270
	(2.6-13.4)	(0.5-2.0)	(40-190)	(40-1280)
p-value	0.04	0.51	0.07	0.06

Table 6: Median IgG1-4 concentration:albumin concentration ratio (interquartile range in parentheses) for young and old individuals. p-value is given for comparison of two groups by Mann-Whitney test.

	IgG1	IgG2	IgG3	IgG4
young	2.3 (1.3-3.5)	0.19 (0.12-0.56)	0.011 (0.007-0.025)	0.09 (0.043-0.225)
old	1.5 (0.7-3.2)	0.23 (0.12-0.58)	0.025 (0.015-0.033)	0.079 (0.006-0.230)
p-value	0.22	0.71	0.001	0.35

Table 7: Median A2-M, LF and albumin concentrations (interquartile range in parentheses) for young and old individuals. p-value is given for comparison of two groups by Mann-Whitney test.

	μg/ml		mg/ml
	A2-M	LF	ALBUMIN
young	125 (62-217)	275 (125-471)	6.1 (1.7-12.4)
old	150 (93-337)	169 (92-289)	4.8 (1.9-8.1)
p-value	0.15	0.05	0.27

Table 8: Time progression points for IgG1-4, A2-M, LF and albumin median concentrations during the course of experimental gingivitis for old and young individuals.

	day 0	day 7	day 14	day 21	day 28
IgG1young (mg/ml)	4.1	11.9	11.4	9.9	6.3
IgG1 old(mg/ml)	3.5	8.5	3.7	4.0	4.7
IgG2 young (µg/ml)	100	780	833	1071	2837
IgG2 old(µg/ml)	917	1211	614	1056	966
IgG3 young (µg/ml)	33	62	100	56	92
IgG3 old(µg/ml)	110	126	113	63	85
IgG4 young (µg/ml)	150	746	1300	544	385
IgG4 old(µg/ml)	233	554	457	324	48
A2-M young (µg/ml)	71	65	189	85	202
A2-M old(µg/ml)	85	209	280	203	132
LF young(µg/ml)	83	225	450	331	415
LF old(µg/ml)	83	80	186	267	105
albumin young (mg/ml)	1.4	5.7	3.2	5.8	6.5
albumin old (mg/ml)	5.3	6.3	2.9	5.0	3.8

in IgG levels tend to occur after seven days for young subjects, but later and/or to a reduced extent, especially in relation to the median level, for old subjects. LF levels also tend to increase more slowly and to a lesser extent in old than in young subjects. The more marked of these differences are illustrated in Figures 2-4 with statistically significant differences denoted by asterisks (*) ($p<0.05$, Mann-Whitney test).

Figure 2: IgG2 levels

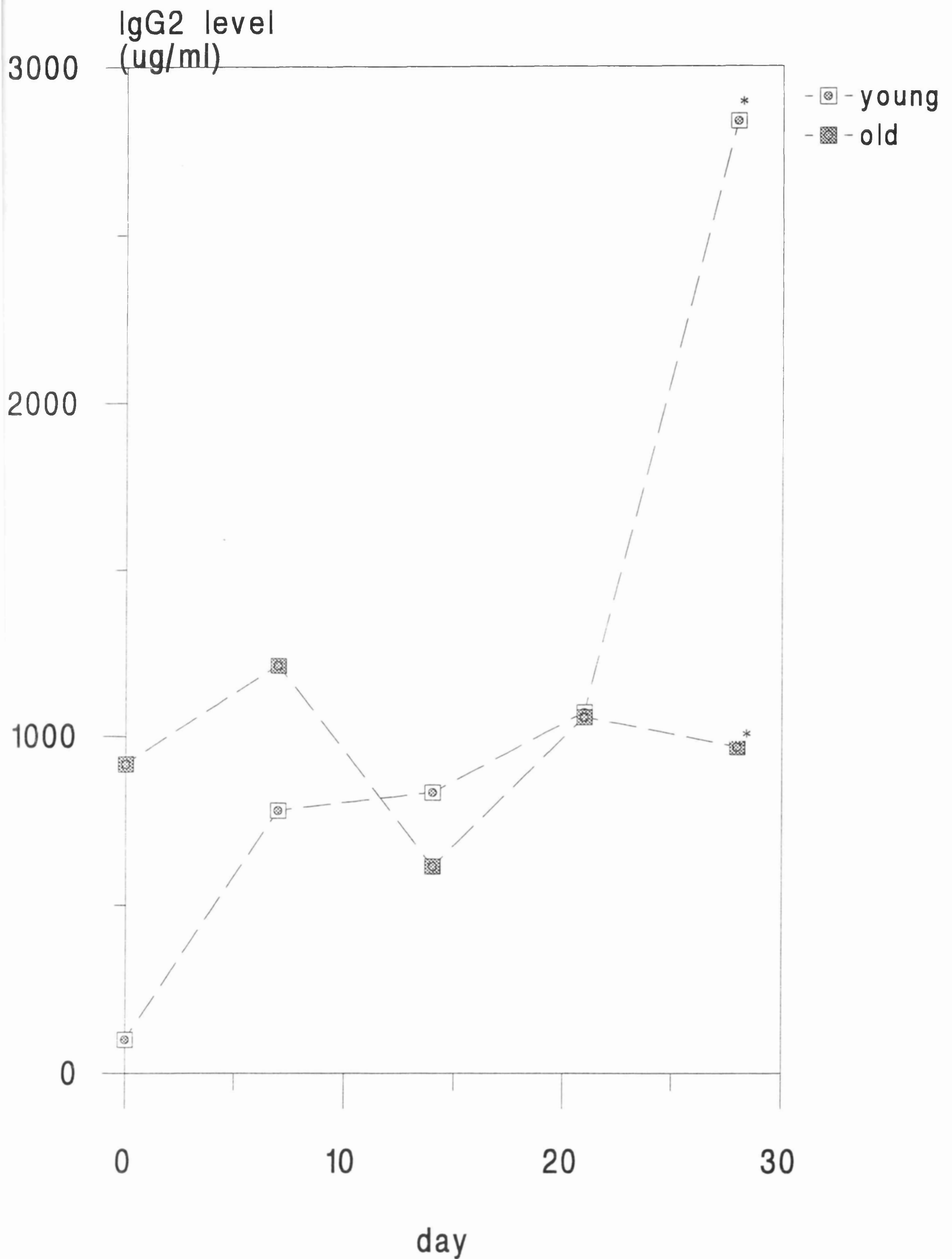


Figure 3: IgG4 levels

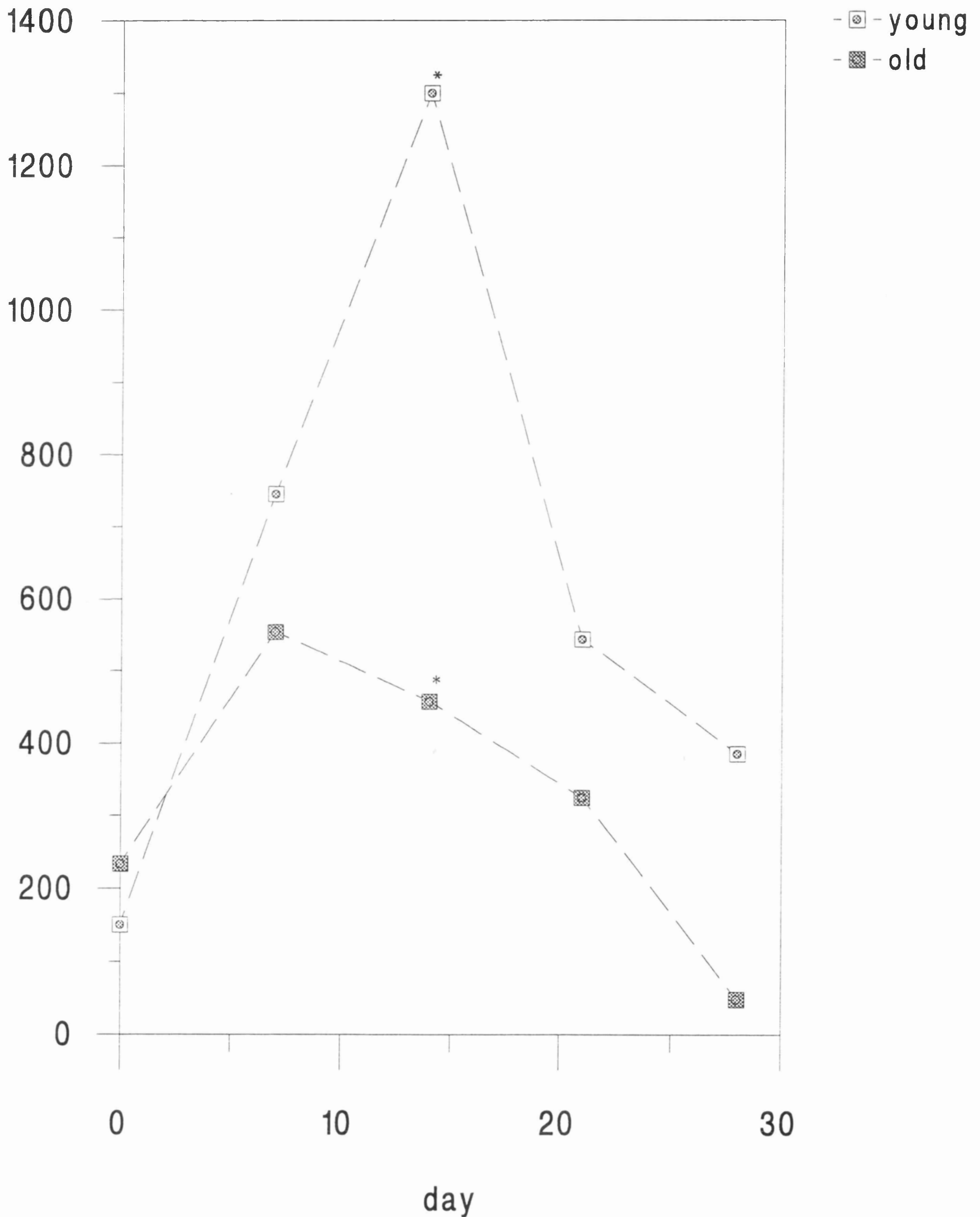
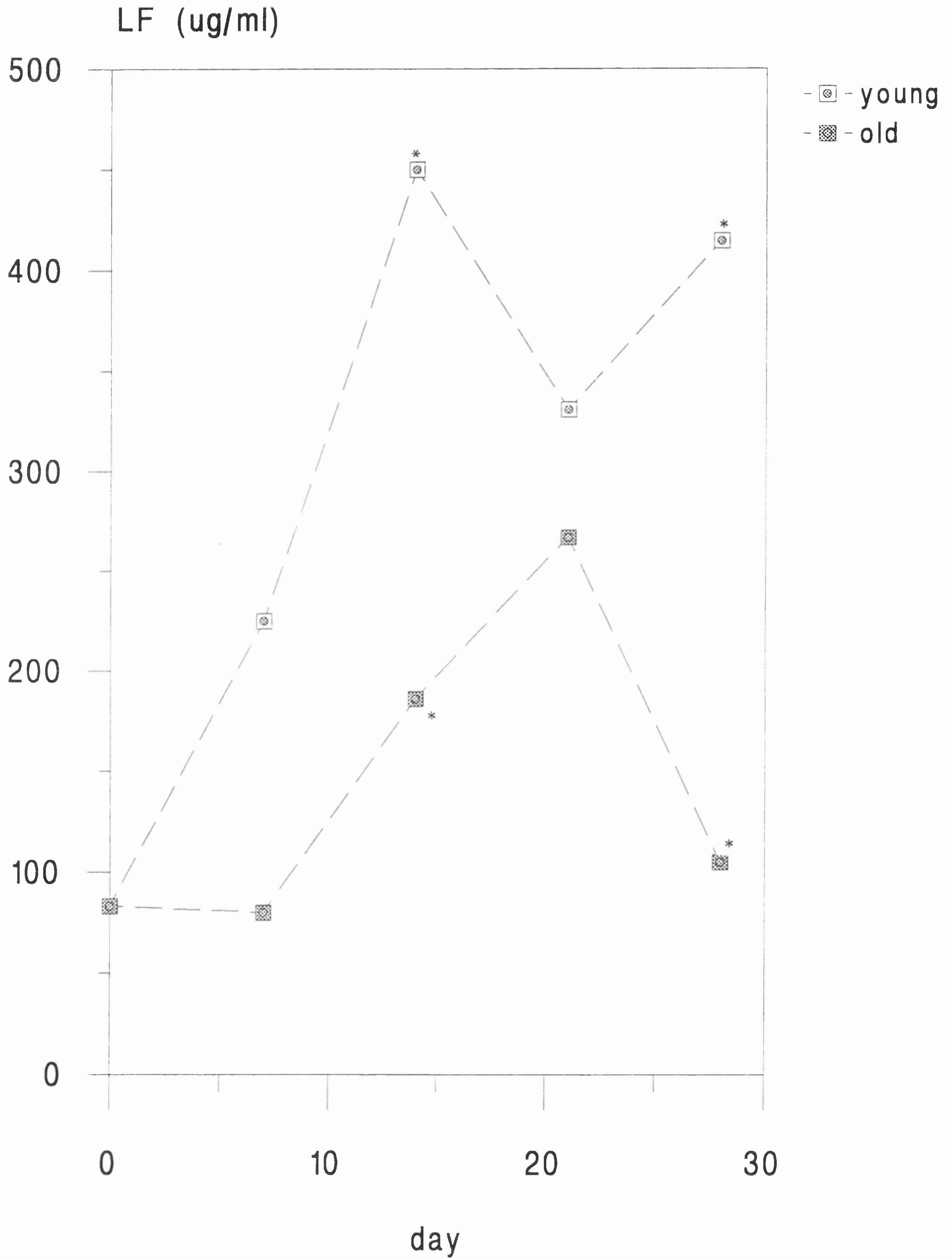


Figure 4: LF levels



3.1.3 Matched comparison of humoral immune response at tooth and implant sites

At the outset a paired comparison between GCF and PISF volumes was carried out using the paired t-test assuming the null-hypothesis, i.e. that there is no difference between these volumes. This established that there was no significant difference between GCF (0.46μl (0.28-0.66)) and PISF (0.52μl (0.36-0.60)) volumes and that, therefore, any observed differences between GCF and PISF are not attributable simply to differences in volume. The comparison between matched teeth and implants in terms of IgG, IgM and IgA antibody titre to *P. gingivalis* and *A. actinomycetemcomitans* are shown in Tables 9 and 10. The antibody titres were non-normally distributed, therefore a Wilcoxon signed rank test was used to determine whether the matched pair values for teeth and implants were significantly different. There is a tendency towards titres being lower in implant sites than in tooth sites (p-value ranging from 0.08 to 0.14), although this failed to be statistically significant at $p<0.05$.

Table 9: Comparison between matched teeth and implants in terms of titre of IgG, IgM and IgA (EU/ml) to *P. gingivalis*. Median values are given with interquartile ranges. The p-value for the Wilcoxon signed rank test using the null hypothesis (i.e. that these values are not different) is given.

	Median titre (EU/ml)		
	Teeth	**Implants**	**p-value**
IgG	315	254	0.14
	(165-899)	(161-348)	
IgM	242	182	0.12
	(138-368)	(136-269)	
IgA	421	383	0.49
	(206-576)	(268-486)	

Table 10: Comparison between matched teeth and implants in terms of titre of IgG, IgM and IgA (EU/ml) to *A. actinomycetemcomitans*. Median values are given with interquartile ranges. The p-value for the Wilcoxon signed rank test using the null hypothesis (i.e. that these values are not different) is given.

	Median titre (EU/ml)		
	Teeth	**Implants**	**p-value**
IgG	280	225	0.08
	(156-394)	(134-310)	
IgM	508	350	0.10
	(218-1015)	(206-866)	
IgA	428	339	0.14
	(195-854)	(232-730)	

Using the same analyses, Tables 11 and 12 demonstrate that, although RCE ratios are in all cases higher at implant sites than at matched natural tooth sites, suggesting greater local production of specific immunoglobulin, the paired comparisons do not yield statistically significant differences. Levels of local production of antibody to *A. actinomycetemcomitans* seem to be much higher than to *P. gingivalis*, perhaps reflecting the relative importance of *A. actinomycetemcomitans* as an opportunistic pathogen in the early stages of disease.

Table 13 shows the correlations between RCE ratios for different immunoglobulin class responses to *P. gingivalis* and *A. actinomycetemcomitans* for natural teeth, and Table 14 provides the same information for implants. An interesting finding here is that, although there are strong correlations between class responses in tooth sites, these are not found in implant sites.

Analyses were performed to examine for correlations between RCE ratios of the same immunoglobulin class to *P. gingivalis* and *A. actinomycetemcomitans* between matched teeth and implants (Table 15). IgG and IgM class responses do not correlate between teeth and matched

Table 11: Comparison between matched teeth and implants in terms of RCE ratio of IgG, IgM and IgA for *P. gingivalis*. Median values are given with interquartile ranges. The p-value for the Wilcoxon signed rank test using the null hypothesis (i.e. that these values are not different) is given.

	RCE		
	Teeth	**Implants**	**p-value**
IgG	0.30 (0.18-0.90)	0.50 (0.23-0.88)	0.23
IgM	1.17 (0.48-2.20)	1.51 (0.85-3.49)	0.31
IgA	1.28 (0.22-5.80)	1.56 (0.62-7.49)	0.44

Table 12: Comparison between matched teeth and implants in terms of RCE ratio of IgG, IgM and IgA for *A. actinomycetemcomitans*. Median values are given with interquartile ranges. The p-value for the Wilcoxon signed rank test using the null hypothesis (i.e. that these values are not different) is given.

	RCE		
	Teeth	**Implants**	**p-value**
IgG	3.09	4.09	0.26
	(1.50-8.31)	(1.81-14.71)	
IgM	11.63	20.83	0.42
	(7.89-36.83)	(9.66-46.93)	
IgA	6.90	11.40	0.59
	(1.80-32.50)	(3.60-68.10)	

Table 13: Correlations between immunoglobulin RCE ratios for *P. gingivalis* and *A. actinomycetemcomitans* in natural tooth sites.

	P. gingivalis		*A. actinomycetemcomitans*	
Correlation	**R^2**	**p-value**	**R^2**	**p-value**
IgG vs IgA	67.9%	<0.001	91.8%	<0.001
IgG vs IgM	15.9%	0.08	79.1%	<0.001
IgM vs IgA	3.0%	0.47	82.7%	<0.001

Table 14: Correlations between immunoglobulin RCE ratios for *P. gingivalis* and *A. actinomycetemcomitans* in implant sites.

	P. gingivalis		*A. actinomycetemcomitans*	
Correlation	R^2	**p-value**	R^2	**p-value**
IgG vs IgA	4.0%	0.40	2.6%	0.50
IgG vs IgM	0.6%	0.74	21.0%	0.04
IgM vs IgA	4.6%	0.36	9.2%	0.19

Table 15: Correlations between immunoglobulin RCE ratios of matched teeth and implants.

	P. gingivalis		*A. actinomycetemcomitans*	
Class	**R^2**	**p-value**	**R^2**	**p-value**
IgG	3.2%	0.45	2.0%	0.55
IgM	12.1%	0.13	0.1%	0.89
IgA	22.6%	0.03	28.1%	0.02

implants, but there are statistically significant correlations between IgA responses.

Overall, these results show that specific antibody titres tend to be lower in implants than in matched natural teeth. This trend fails to achieve statistical significance, but does suggest that a larger follow-up study would be worthwhile. The data presented in tables 13-15 are indicative of differences in the correlations between rates of local production of specific immunoglobulins between matched teeth and implants, suggesting possible differences in the nature and proportions of the plasma cell infiltrate between implants and natural teeth. This finding is also deserving of further study.

3.1.4 Checkerboard immunoblotting of GCF

This technique was demonstrated here to have the potential to screen a large number of GCF samples against a large number of bacterial antigens. 56 GCF x 10 antigens were possible simultaneously. An example is shown in Figure 5. However, some purified antigens e.g. the ARG-1 protease did not coat onto the membrane even at relatively high concentration. Moreover, the data produced were qualitative making it difficult to correlate levels in different sites. In general, however, antibody levels to *P. gingivalis* seemed to be lower in deeper pockets than in shallower pockets within the same patient; bearing out the findings of section 3.1.1.

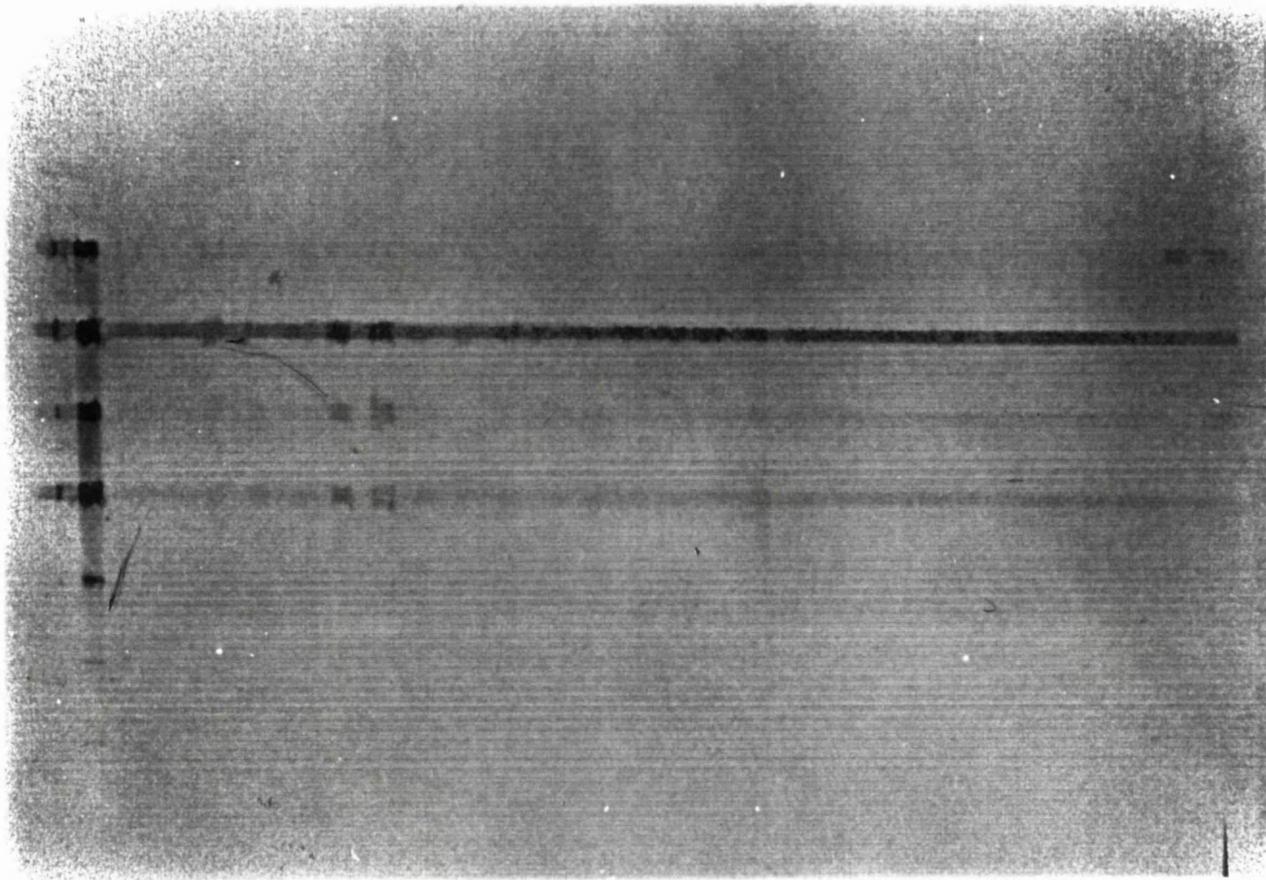


Figure 5: Illustration of typical checkerboard immunoblot.

3.2 In situ hybridisation studies in plasma cells

3.2.1 Study of κ/λ-producing plasma cells

The connective tissue subjacent to the pocket epithelium and central zone of the lamina propria revealed a dense and diffuse infiltration of lymphocytes and dilated capillaries. All specimens exhibited clustering of plasma cells mainly in the perivascular areas deep within the central zone of the lamina propria and subjacent to the epithelium. Plasma cells and small lymphocytes (presumably B cells) showed weak to strong cytoplasmic staining with either κ or λ probes (Figure 6 and 7). The specificity of the hybridisation signals were confirmed by the control sections which were incubated with a hybridisation solution lacking probe and showed a complete absence of signal in plasma cells (Figure 8). In addition, results of the hybridisation staining procedure following pre-treatment with RNAse were negative,

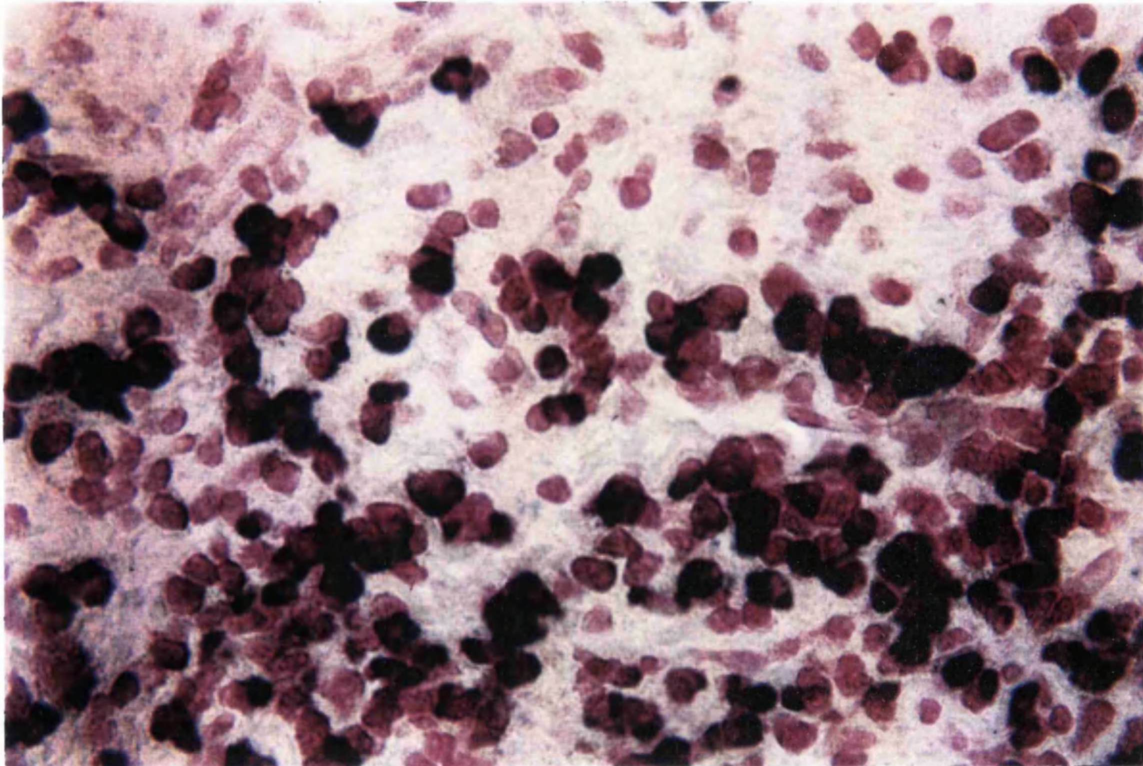


Figure 6: Slide showing κ-positive plasma cells.

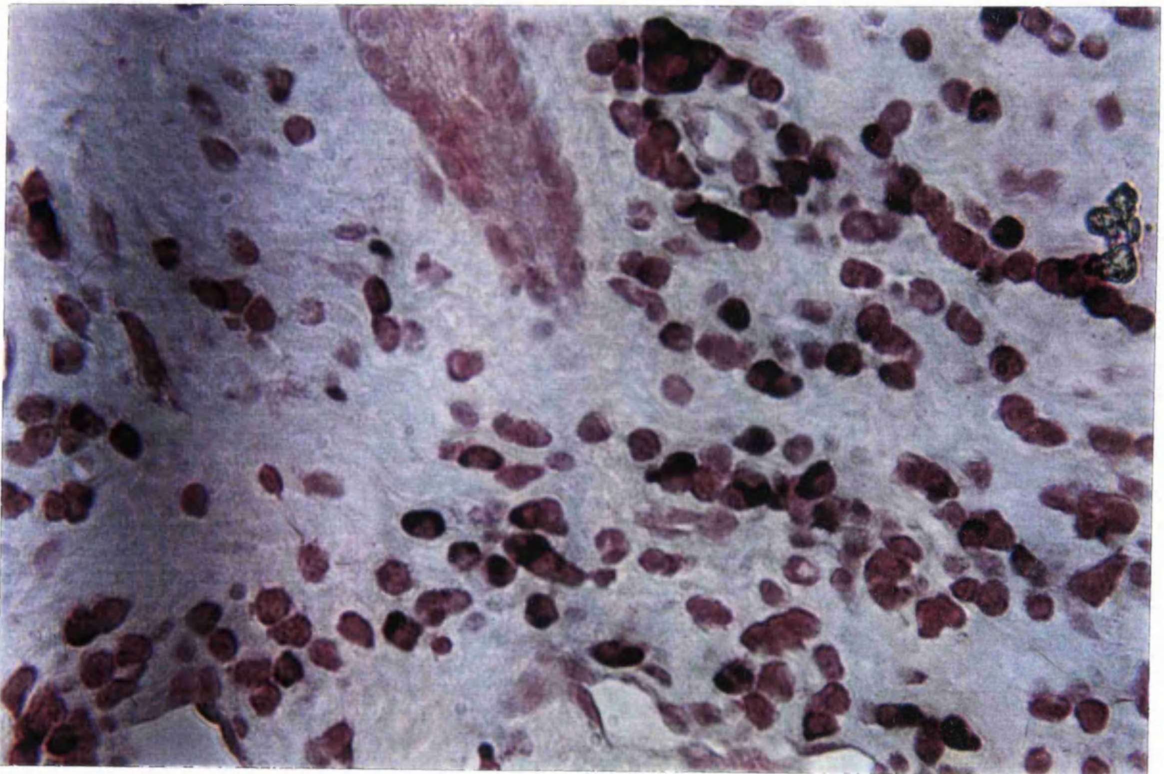


Figure 7: Slide showing λ-positive plasma cells.

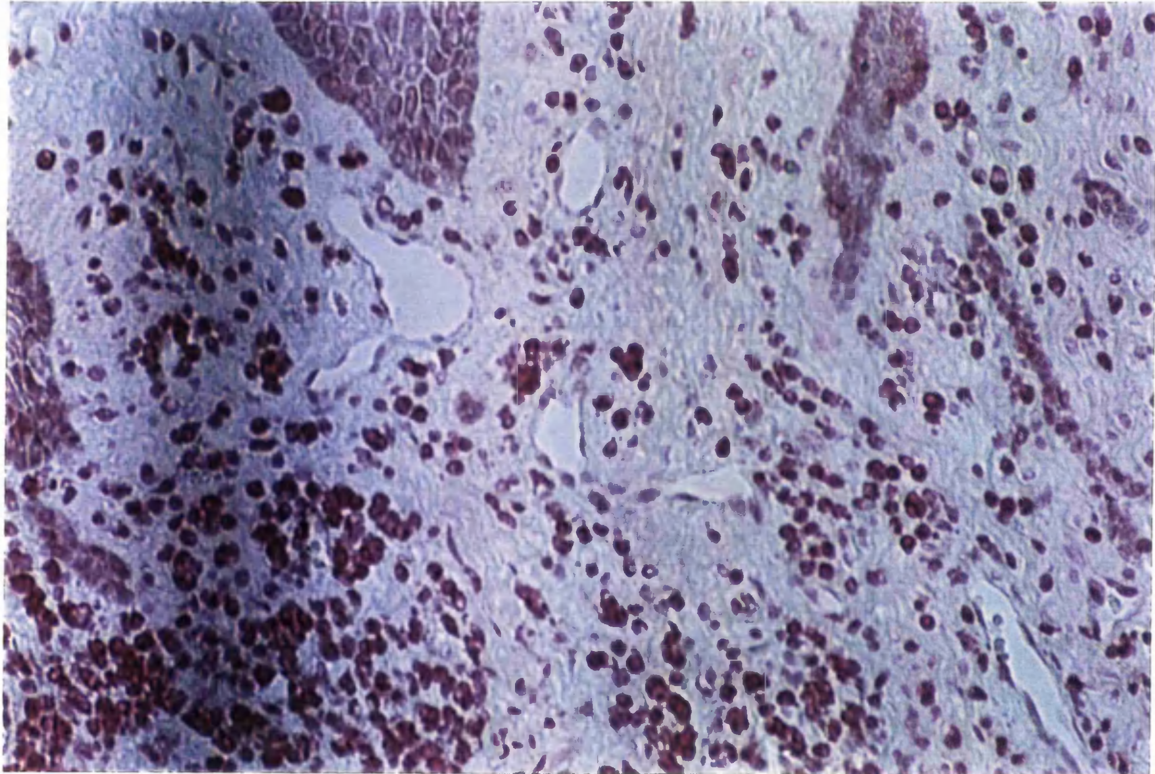


Figure 8: Slide showing negative control.

confirming that the method specifically indicates the presence of RNA.

κ chain positive plasma cells were the most predominant in all of the sections, and this difference was statistically significant ($p < 0.01$). Mean percentages calculated from these data were 67.5 ± 9.5 % κ (53 - 82.8 %) and 32.5 % λ (17.2 - 47 %) producing cells. The ratio of κ to λ chain positive cells ranged from 1.1 to 4.8 (mean = 2.4 ± 1.1 SD). Table 16 indicates the number of biopsies per patient and shows both mean site and subject percentages of κ light chain positive plasma cells. Considering the subject as the analytical unit, the mean percentage of patient plasma cells with the κ light chain is 70.4% (SD ±7.0%), and the range between patients is 60-79%.

Table 16. Patient details, site pocket depths and percentages of plasma cells which were positive for κ light chains. Means and Standard Deviations (SD) are given for each subject.

Subject (sex)	Age	PD (mm)	%κ plasma cells	Meanκ% for subject	Subject SD
a(M)	55	5	78.7%	78.7%	
b(M)	41	7	79.3%	79.3%	
c(F)	34	5	61.7%	63.8%	± 0.13
		4	64.3%		
		5	65.1%		
d(M)	41	5	67.8%	68.3%	± 0.06
		5	68.7%		
e(M)	38	5	54.2%	60.0%	± 0.44
		6	64.4%		
f(F)	19	3	82.8%	75.6%	± 1.85
		6	76.5%		
		7	53.0%		
g(F)	42	6	68.7%	68.8%	
h(M)	45	6	53.7%	68.8%	± 1.04
		6	58.2%		
		5	76.1%		
		5	74.6%		

3.2.2 Study of local production of IgG and IgA

Preliminary experiments verified the specificity of the IgG and IgA subclass-specific probes. In all gingival biopsy tissues, cytoplasmic staining was observed in plasma cells for IgG and IgA subclass probes in serial sections. The majority of immunoglobulin positive cells were observed in the lamina propria and some cells were identified subjacent to the epithelium. Specificity of the hybridization signals was confirmed by the control results. The negative control sections incubated with a hybridization solution without probe or sense probe, showed a complete absence of signal in plasma cells and lymphocytes, in all cases. Further results of the hybridization staining procedure following treatment with RNAse were negative, confirming that the method specifically indicates the presence of RNA (data not shown). In addition, the hybridization of the labelled probes was greatly reduced or abolished when the concentration of unlabelled probe against the same subclass was five- or ten-fold in excess. In contrast,

the hybridization of the labelled probes was unaffected when the concentration of unlabelled probe against the other subclass was five- or ten-fold in excess.

The major IgG subclass mRNA expressing cells was IgG1 (mean 63%) followed by IgG2 (23%) of total IgG plasma cells and IgG3 and IgG4 were present to a much lesser extent (3% and 10% respectively). Similar proportions of IgG subclass proteins were detected in GCF as determined by ELISA (Table 17). The relative proportions of IgG3 and IgG4 varied somewhat between GCF and biopsy plasma cells but these differences were not statistically significant. Serum levels of IgG subclasses were not measured in this study.

IgA1 mRNA-expressing cells were predominant (65.1%) than IgA2 mRNA-expressing cells and this difference was statistically significant ($p < 0.0001$) but evenly distributed IgA2 mRNA-expressing cells (i.e. %IgA2 between 45 and 50%) could be also seen in 5/24 cases. IgA1/IgA2 ratio varied widely (median 1.67, range 1.03 - 10). Gingival tissue sections used in this study gave the strongest hybridization signals with a proteinase K concentration of 30-50 mg/ml and showed good morphological detail at this concentration, while the proteinase K

Table 17. Immunoglobulin G and A subclasses distribution for GCF, serum immunoglobulins and tissue plasma cell mRNA. Concentration (µg/ml) with standard deviations and percentages of total IgG or total IgA, are given for GCF. For the biopsy tissue, mean cell numbers represent the mean number per section, with standard deviation and percentage of total IgG mRNA positive plasma cells.

	IgG1	**IgG2**	**IgG3**	**IgG4**	**IgA1**	**IgA2**
GCF	10553 (14452) 66%	4998 (7901) 31%	118 (111) 1%	313 (503) 2%	1024 (2558) 21%	6536 (11105) 79%
Biopsy (mRNA +ve)	1007 (114) 63%	367 (60) 23%	40 (46) 3%	167 (224) 10%	151 (274) 65%	85 (135) 35%

digestion at less than 10 mg/ml gave very weak or no signal. Counterstaining with 1% neutral red gave better contrast than haematoxylin (blue color) with the ISH staining, as the precipitation colour of NBT with BCIP is blue black.

IgA1 (1024 ± 2558 mg/ml) and IgA2 (6536 ± 11105 mg/ml) proteins were detected in all GCF samples (IgA1<IgA2, $p=0.009$). The proportion of IgA subclass proteins in GCF samples is shown in Table 17 (IgA1 = 21%; IgA2 = 79%). IgA1 Fab fragments (1989 ± 2781 mg/ml) were higher than intact IgA1 but this was not statistically significantly ($p = 0.09$). However, when correction was made for the molar ratio of Fab:intact IgA of approximately 3.3:1, then this became significant at $p<0.05$. There was no significant correlation in the amounts of IgA1 and IgA2 proteins in GCF samples with the numbers of their respective IgA subclass mRNA-expressing cells taken from the same site ($r = -0.1383$, -0.0972; r = Pearson's correlation coefficient), respectively. An example of double staining with IgG and IgA probes is shown in Figure 9. Figure 10 shows detection of IgG1 and IgG2. IgG3 and IgG4 are demonstrated in Figure 11.

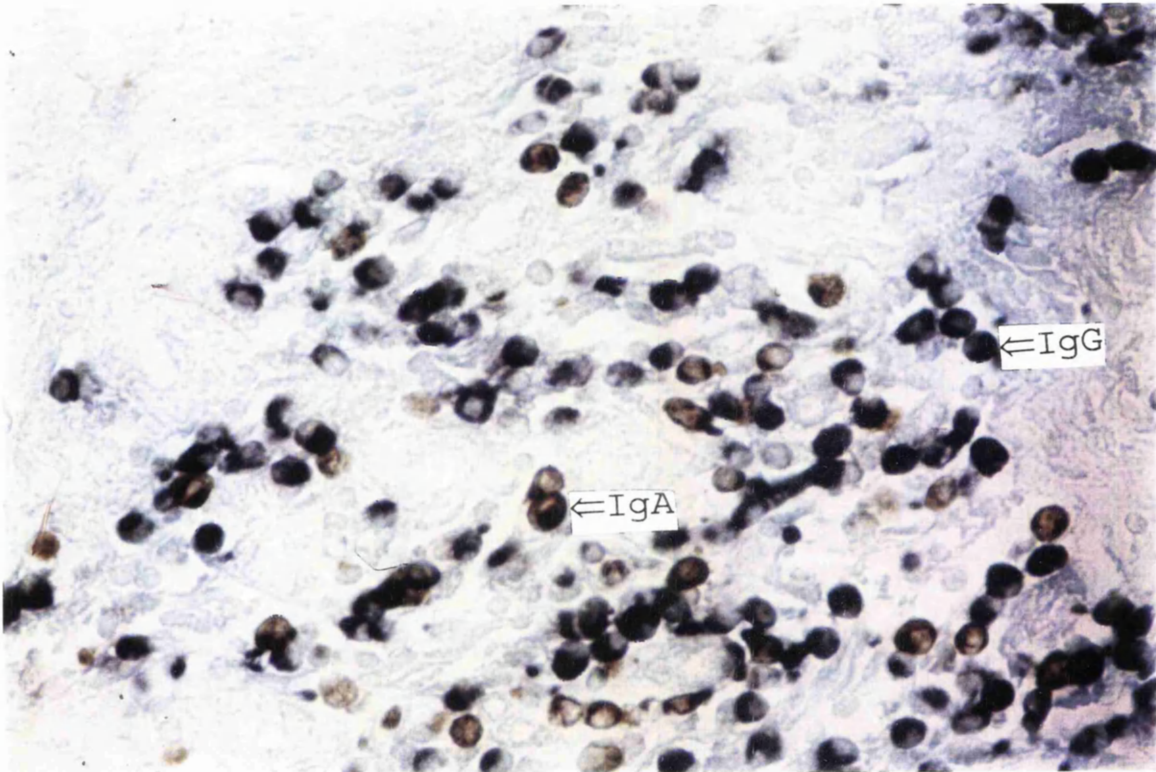
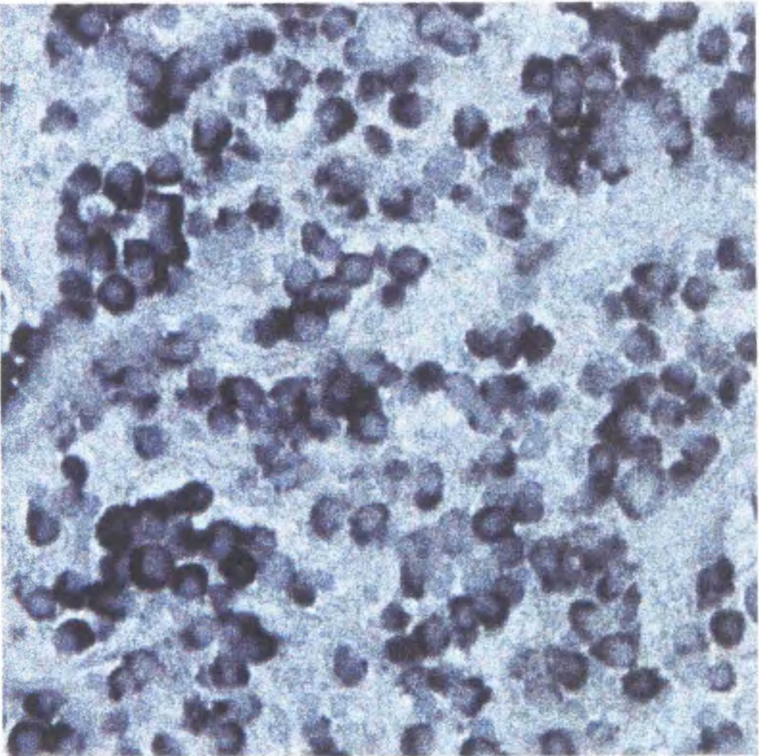
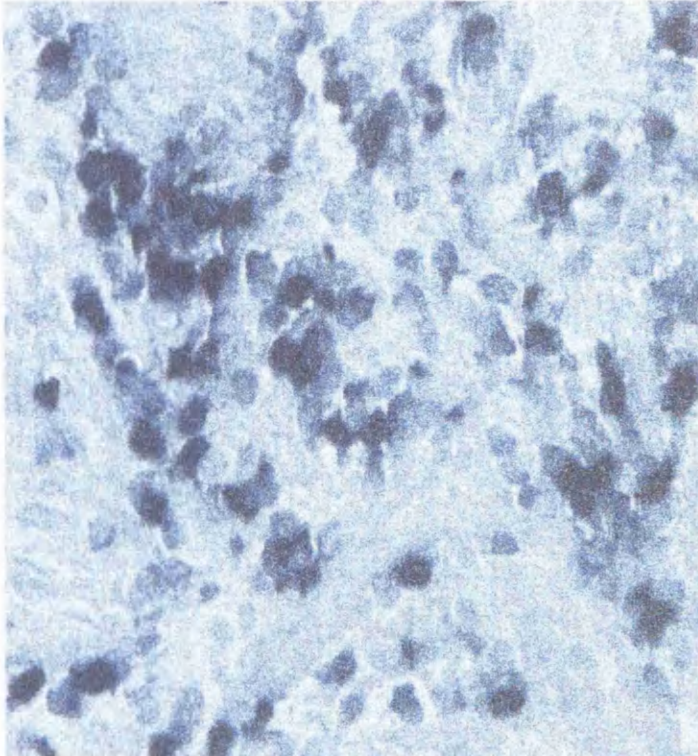


Figure 9: Slide showing double staining for IgG and IgA.

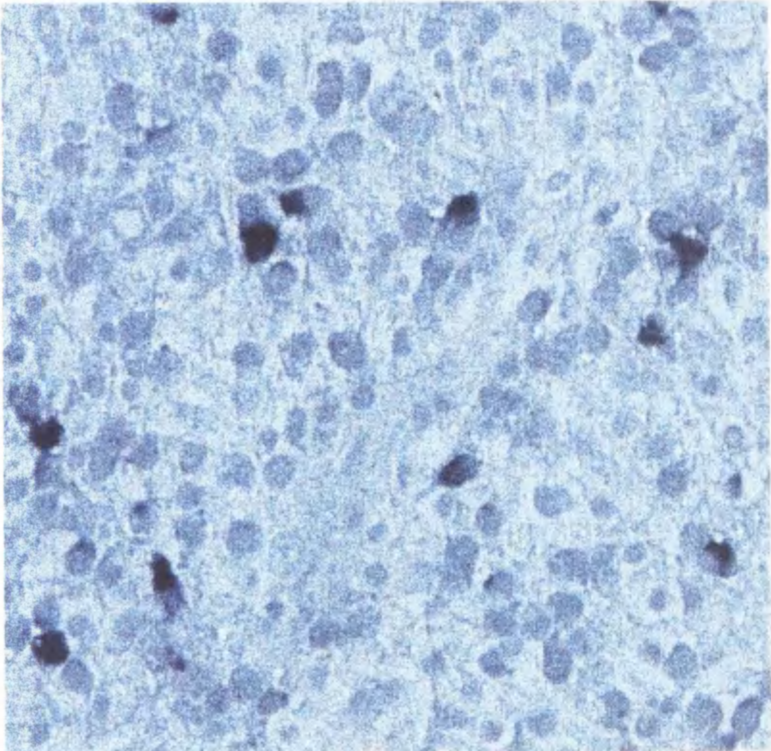


A

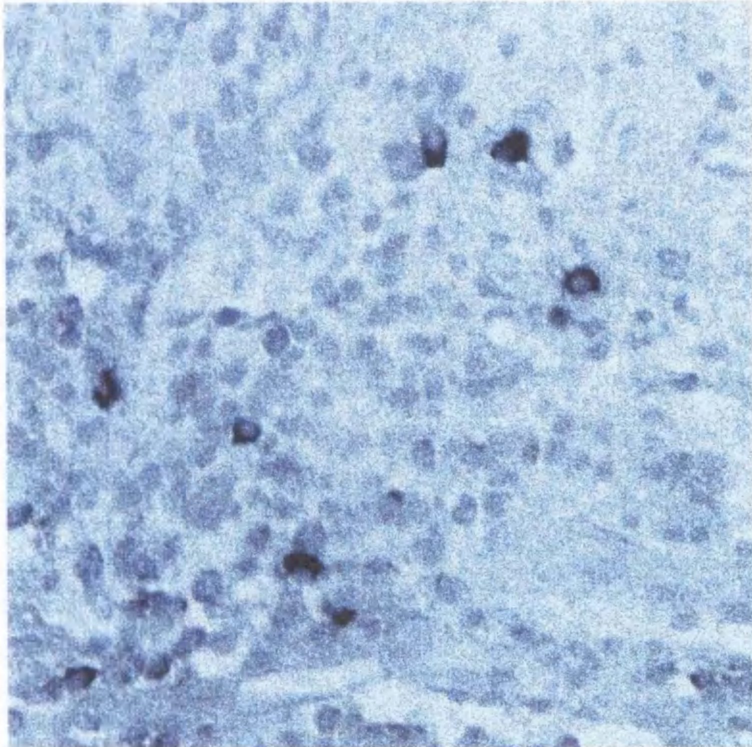


B

Figure 10: Slides showing plasma cells positive for IgG1 (A) and IgG2 (B).

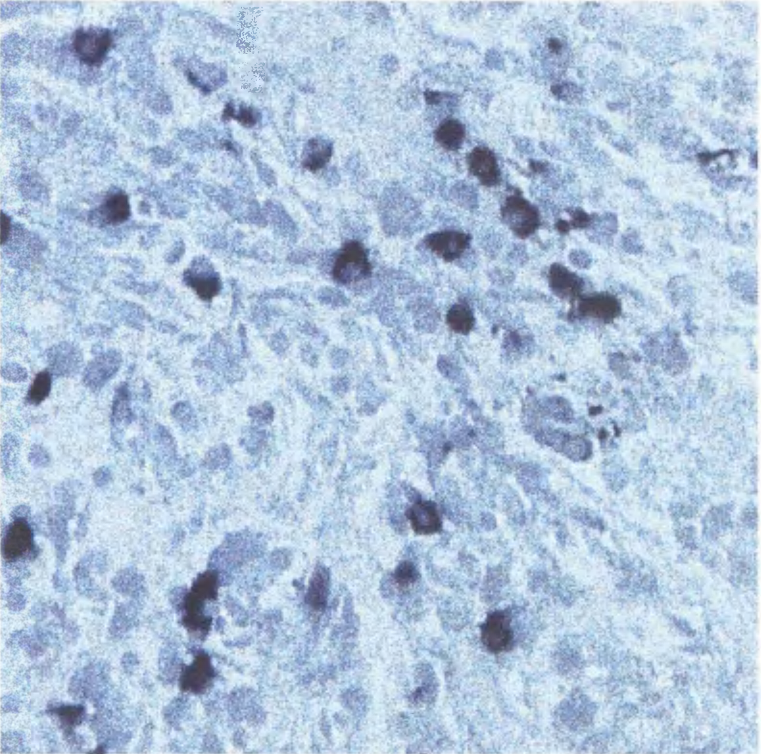


A

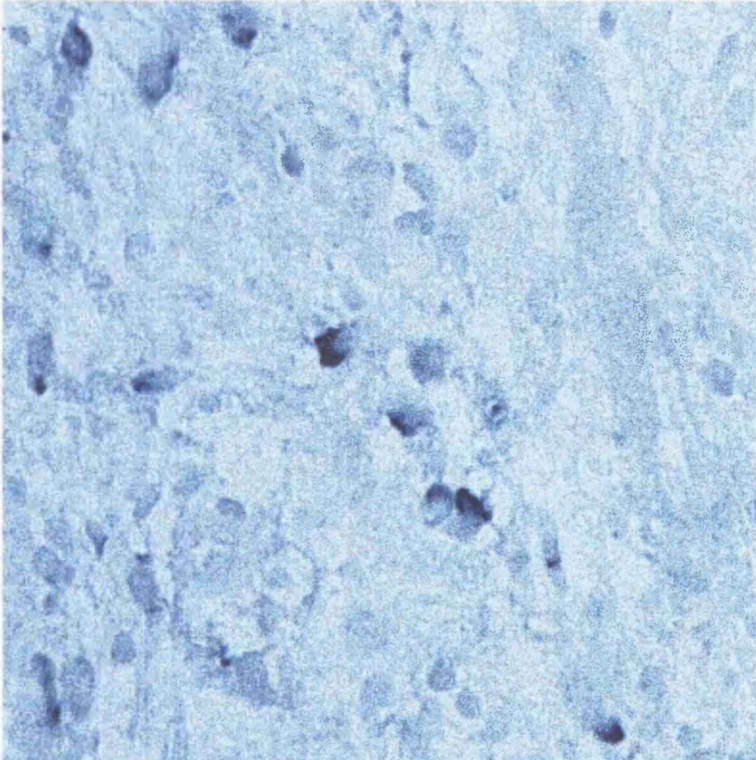


B

Figure 11: Slides showing plasma cells positive for IgG3 (A) and IgG4 (B).



A



B

Figure 12: Slides showing plasma cells positive for IgA1 (A) and IgA2 (B).

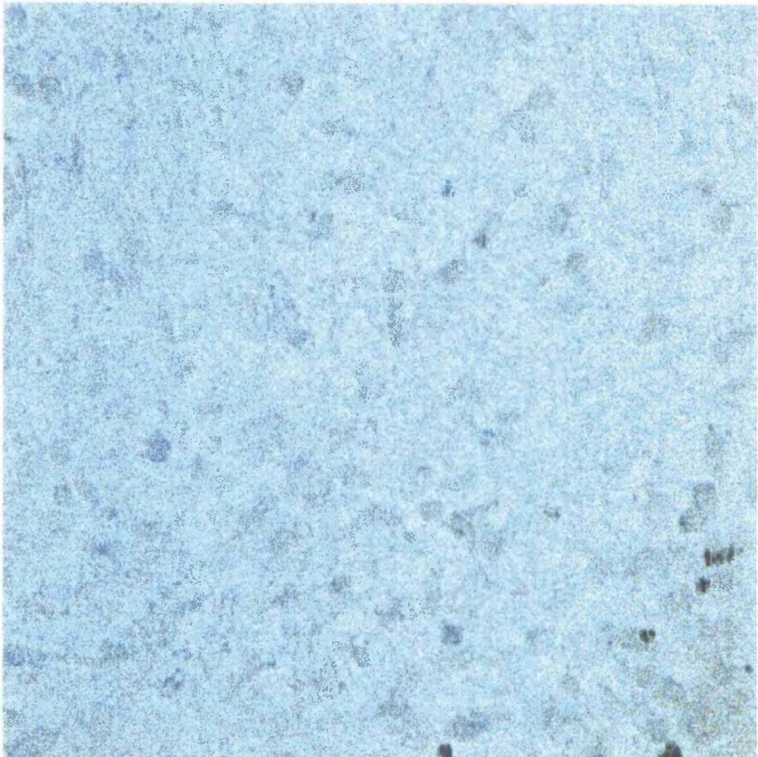


Figure 13: Slide showing typical example of negative control staining using sense probes (in this case for Dig-IgG and biotin-IgA).

Figure 12 shows IgA1 and IgA2. Negative controls for IgG and IgA are shown in Figure 13.

Table 18: Isolation of *Pg* and *Aa* from test sites in ten subjects with refractory periodontitis, sampled at (i) baseline; (ii) after 52 weeks and; (iii) following antibiotic therapy at 75 weeks.

Patient	Number of sites positive/sites sampled per patient					
	Baseline		week 52		week 75	
no.	*Pg*	*Aa*	*Pg*	*Aa*	*Pg*	*Aa*
1	7/9	9/9	6/9	8/9	0/9	0/9
2	9/9	0/9*	0/9	0/9	0/9	0/9
3	3/3	3/3	0/3	3/3	0/3	3/3
4	6/6	0/6*	6/6	6/6	0/6	2/6
5	6/6	6/6	2/6	4/6	0/6	2/6
6	6/6	0/6	3/6	0/6	0/6	0/6
7	1/6	3/6	0/6	2/6	0/6	0/6
8	0/6	4/6	1/6	2/6	0/6	0/6
9	2/3	0/3*	0/3	0/3	0/3	0/3
10	6/6	0/6	0/6	0/6	0/6	0/6

**Haemophilus aphrophilus* isolated at baseline (see text)

3.3 Studies of serum antibody avidity

3.3.1 Preliminary study of effects of periodontal treatment on antibody avidity and plaque microbiology

The isolation of *A. actinomycetemcomitans* and *P. gingivalis* from each patient during this study at baseline, week 52 and after antibiotic therapy at week 75 is given in Table 18. At baseline all patients harboured one or both of the putative periodontal pathogens under investigation. *A. actinomycetemcomitans* was isolated from five patients at baseline and all five were again positive at week 52. From three further patients at baseline colonies resembling *A. actinomycetemcomitans* were cultured but were found to be catalase negative and were presumptively identified as *Haemophilus aphrophilus*. All three patients had lost this organism by week 52 but one became positive for *A. actinomycetemcomitans* at that time. *P. gingivalis* was isolated from 9/10 patients at baseline. It was isolated from one additional patient by week 52 but five patients

Table 19: Antibody titres (Log_{10} ELISA units) for *Pg* for individual patients at baseline and at week 75

Patient	IgG		IgM		IgA		Culture	
	baseline	wk75	baseline	wk 75	baseline	wk 75	baseline	wk 75
1	3.371	3.185	1.950	1.778	4.183	5.106	+	-
2	3.208	3.195	2.789	2.636	4.604	5.077	+	-
3	3.527	3.262	2.628	2.513	5.319	6.499	+	-
4	2.633	2.802	2.474	2.576	6.566	7.018	+	-
5	2.590	2.623	5.095	4.675	3.140	3.163	+	-
6	3.063	3.044	3.545	3.622	7.840	7.454	+	-
7	3.175	3.187	3.768	3.618	3.288	2.973	+	-
8	2.423	2.779	3.723	3.417	3.782	3.532	-	-
9	2.500	3.641	4.437	4.437	6.823	5.918	+	-
10	3.617	3.706	1.792	4.220	4.292	5.360	+	-

Table 20: Antibody titres (Log_{10} ELISA units) for *Aa* for individual patients at baseline and at week 75

Patient	IgG		IgM		IgA		Culture	
	baseline	wk75	baseline	wk 75	baseline	wk 75	baseline	wk 75
1	4.565	4.053	2.262	1.240	7.070	5.674	+	+
2	3.346	3.447	2.340	2.314	4.831	6.324	-	-
3	3.431	2.695	2.719	2.940	4.144	5.826	+	+
4	2.382	4.075	3.015	3.450	3.372	5.702	-	+
5	3.149	3.032	2.993	2.932	3.943	3.583	+	+
6	2.338	2.401	2.556	2.883	4.000	3.334	-	-
7	6.025	5.901	5.224	4.881	6.399	5.144	+	-
8	3.386	3.186	4.153	3.278	5.302	3.445	+	-
9	4.936	4.654	4.980	4.688	5.192	3.736	-	-
10	2.899	2.976	2.428	2.179	3.454	3.940	-	-

appeared to have lost *P. gingivalis* from all sites sampled after the hygiene and surgical phase of the study. Following antibiotic therapy and re-sampling at week 75, *P. gingivalis* was not isolated from any site in any patient. Three subjects, however, from whom *A. actinomycetemcomitans* was isolated at week 52 yielded *A. actinomycetemcomitans* again at week 75. Two of these patients had the organism at baseline and from the third, *H. aphrophilus* was isolated at baseline. The degree of growth of *A. actinomycetemcomitans* declined, however, in all sites and the number of sites in these three patients from whom *A. actinomycetemcomitans* was isolated declined from a total of 13/15 to 7/15.

Individual patient antibody titres, expressed as $\log_{10}$ ELISA units, at baseline and after treatment are given for *P. gingivalis* in Table 19 and for *A. actinomycetemcomitans* in Table 20. There was a wide variation in antibody titres and for *P. gingivalis* (Table 19) there was no correlation between the change in titre and the loss of the organism on culture. Indeed for IgG and IgA, six subjects showed a rising titre by week 75 compared with baseline. Most subjects showed a decline in antibody titre to *A. actinomycetemcomitans*

Table 21: Titres and avidities of antibodies (IgG, IgM and IgA) to *Pg* and *Aa* for 10 patients at baseline and at week 75. Median differences and interquartile ranges are given for increases (+) or decreases (-) between baseline and week 75. Significant differences using the Wilcoxon signed rank test and the null hypothesis (i.e. that there is no difference) are highlighted by * ($p<0.033$).

Antibody	median titre diff	inter-quartile range	median avidity diff	inter-quartile range
Pg IgG	+37	-242/+486	-0.01	-0.16/+0.27
Pg IgM	-141	-4699/+51	+0.08	-0.06/+0.20
Pg IgA	-462	$->10^5/+>10^5$	-0.03	-0.23/+0.15
Aa IgG	-616	-29333/+244	+0.08	-0.04/+0.230
Aa IgM	-123	-20917/+361	+0.09	-0.01/+0.20
Aa IgA	-6388	$->10^5/+>10^5$	+0.01	-0.10/+0.32

Table 22: Titres and avidities of antibodies (IgG,IgM and IgA) to *Pg* and *Aa* for 10 patients at baseline compared with matched controls. Median differences and interquartile ranges are given for increases (+) or decreases (-) of patients over matched controls. Significant differences using the Wilcoxon signed rank test and the null hypothesis (i.e. that there is no difference) are highlighted by * ($p<0.033$).

Antibody		median titre diff	inter-quartile range	median avidity diff	inter-quartile range
Pg	IgG	+93	-3885/+1435	+0.50*	+0.34/+0.87
Pg	IgM	+77	-1020/+8573	+0.34*	+0.18/+0.74
Pg	IgA	+26968*	+4285/+>10^5	+0.90*	+0.02/+1.42
Aa	IgG	+2003	-10/+46280	+0.40*	+0.15/+0.68
Aa	IgM	+38	-98/+32192	-0.03	-0.14/+0.09
Aa	IgA	+13051	-1759/+>10^5	0.00	-0.50/+0.46

Table 23: Titres and avidities of antibodies (IgG, IgM and IgA) to *Pg* and *Aa* for 10 patients after treatment (week 75) compared with matched controls. Median differences and interquartile ranges are given for increases (+) or decreases (-) of patients over matched controls. Significant differences using the Wilcoxon signed rank test and the null hypothesis (i.e. that there is no difference) are highlighted by * ($p<0.033$).

Antibody	median titre diff	inter-quartile range	median avidity diff	inter-quartile range
Pg IgG	+210	-4258/+1418	+0.53*	+0.32/+0.89
Pg IgM	+28	-1092/+6386	+0.51*	+0.05/+0.80
Pg IgA	+170196*	+2321/+>10^6	+0.84*	+0.10/+1.53
Aa IgG	+1751	-10/+17331	+0.77*	+0.18/+0.83
Aa IgM	-130	-661/+13491	+0.05	-0.05/+0.17
Aa IgA	+67209	-11995/+>10^5	-0.02	-0.31/+0.32

when the organism was lost on culture. Subject no. 4, that gained *A. actinomycetemcomitans* during the study showed a rise in specific antibody titre in each antibody class (Table 20).

Table 21 shows the differences in median titres of each class of antibody to *P. gingivalis* and *A. actinomycetemcomitans* and differences in median avidities when comparing the baseline serum samples from the post-treatment samples obtained at week 75. The changes over the treatment period were rather small and never significant. The median avidities for each class of antibody to *A. actinomycetemcomitans* declined following treatment, almost reaching significance for IgM. Table 22 shows the differences in median antibody titres and avidities obtained when comparing the baseline sample from the 10 patients with the matched control samples and Table 23 shows the differences in median antibody titres and avidities obtained when comparing the post-treatment patient samples with the control samples. There was no significant fall in median antibody titre nor in median antibody avidity to either organism from baseline to week 75, following therapy and antibiotic administration. When compared with matched

controls at baseline (Table 22) and week 75 (Table 23), however, significantly higher values were obtained for the patients than controls with respect to avidity to: (i) all classes of antibody directed against *P. gingivalis* and; (ii) for *IgG* against *A. actinomycetemcomitans*. Median antibody titres to *P. gingivalis* IgA were also significantly higher in patients than in matched controls.

Table 24: Analysis of entire patient group (n=17) giving before and after treatment (HPT) comparison in terms of antibody to *P. gingivalis* and *A. actinomycetemcomitans*. Mean titres are given with interquartile ranges in parentheses. P-values for the comparisons are given for paired t-test (avidity) or Wilcoxon signed rank test (titre).

	P. gingivalis						*A. actinomycetemcomitans*					
	Avidity (ID50)			Titre (EU)			Avidity (ID50)			Titre (EU)		
	Baseline	p	post-HPT	Baseline	p	post-HPT	Baseline	p	post-HPT	Baseline	p	post-HPT
IgG	1.01 (0.68-1.20)	0.05	1.38 (0.70-2.00)	700 (306-4471)	0.62	1375 (518-3176)	0.77 (0.63-0.90)	0.43	0.67 (0.43-0.66)	128 (16-407)	0.02	724 (88-54423)
IgM	0.75 (0.66-0.86)	0.17	0.98 (0.64-1.12)	346 (214-512)	0.92	461 (264-716)	0.49 (0.34-0.62)	0.42	0.53 (0.47-0.64)	198 (103-460)	0.03	461 (140-3803)
IgA	1.27 (1.01-1.44)	0.81	1.22 (0.80-1.58)	89 (30-951)	0.01	237 (149-2750)	0.83 (0.57-1.14)	0.72	0.80 (0.65-0.92)	9 (4-14)	0.02	19 (11-54)

3.3.2 Longitudinal study of clinical and immunological effects of periodontal therapy

An analysis of the entire patient group before and after HPT is given in Table 24. This demonstrates a tendency towards an increase in avidity of IgG to *P. gingivalis* and significant increases in titre to *A. actinomycetemcomitans* after therapy. For comparison only, since this study did not attempt to relate diseased and healthy individuals, the corresponding healthy control data for IgG, IgA and IgM avidity (mean, interquartile range) and titre (median, interquartile range) respectively to these organisms were as follows:-

Pg: IgG; 1.00 (0.69-1.12). 320 (118-587).

IgA; 1.18 (0.60-1.69). 13 (7-30).

IgM; 0.70 (0.56-0.86). 412 (188-1139).

Aa: IgG; 0.81 (0.60-1.00). 749 (171-3217).

IgA; 1.38 (0.75-2.02). 8 (4-153).

IgM; 0.64 (0.38-1.04). 152 (95-298).

Analyses were performed for the effect of treatment on avidity and titre of antibody to the two organisms after dichotomisation of the treatment group into sub-

Table 25: Analysis of treatment effect on IgG, IgM and IgA antibody to *P. gingivalis* in terms of sub-groups which were seropositive or seronegative at baseline. p-value for paired comparison was derived from paired t-test (avidity) or Wilcoxon signed rank test (titre). Interquartile ranges given in parentheses.

		avidity (ID50)				titre (EU)			
		Baseline	post-HPT	p	D	Baseline	post-HPT	p	D
IgG	sero-positive <n=9>	1.14 (0.82-1.44)	1.82 (0.88-2.81)	0.026	+0.69	2559 (1477-48590)	2725 (1066-14502)	0.53	-568
	sero-negative <n=8>	0.85 (0.63-1.09)	0.85 (0.54-1.14)	1.00	0.00	306 (195-547)	518 (293-2000)	0.08	+474
IgM	sero-positive <n=3>	0.76 (0.69-0.87)	0.68 (0.58-0.75)	0.25	-0.08	941 (853-1054)	721 (501-724)	0.18	-288
	sero-negative <n=14>	0.75 (0.66-0.87)	1.05 (0.65-1.24)	0.14	+0.30	290 (186-489)	362 (232-658)	0.45	+31
IgA	sero-positive <n=11>	1.38 (1.00-1.65)	1.21 (0.67-1.56)	0.57	-0.18	602 (89-1226)	625 (196-3488)	0.056	+652
	sero-negative <n=6>	1.06 (0.99-1.14)	1.24 (0.87-1.62)	0.38	+0.18	26 (21-33)	128 (62-2871)	0.036	+12

Table 26: Analysis of treatment effect on IgG, IgM and IgA antibody to *A. actinomycetemcomitans* in terms of sub-groups which were seropositive or seronegative at baseline. p-value for paired comparison was derived from paired t-test (avidity) or Wilcoxon signed rank test (titre). Interquartile ranges given in parentheses. (D = mean difference).

		Avidity (ID50)				Titre (EU)			
		Baseline	post-HPT	p	D	Baseline	post-HPT	p	D
IgG	sero-positive <n=5>	0.73 (0.51-0.99)	0.60 (0.42-0.78)	0.24	-0.14	631 (407-106)	105 (2139-105)	0.79	+3754
	sero-negative <n=12>	0.81 (0.63-0.92)	0.68 (0.42-0.64)	0.45	-0.13	27 (11-138)	391 (27-7291)	0.006	+518
IgM	sero-positive <n=8>	0.53 (0.32-0.76)	0.50 (0.29-0.65)	0.73	-0.03	459 (320-745)	601 (159-7094)	0.29	+914
	sero-negative <n=9>	0.45 (0.34-0.60)	0.56 (0.48-0.65)	0.08	+0.11	118 (69-168)	461 (124-3014)	0.03	+511
IgA	sero-positive <n=11>	0.82 (0.59-1.16)	0.69 (0.64-0.77)	0.59	-0.13	32 (27-37)	20 (16-32)	0.42	-11
	sero-negative <n=6>	0.84 (0.52-1.16)	0.82 (0.62-0.96)	0.89	-0.02	8 (4-10)	18 (10-147)	0.003	+22

Table 27: Analysis of clinical status according to serostatus for *P. gingivalis* and *A. actinomycetemcomitans*. BOP % (mean and SD) and probing depth (mean and SD) are given for seropositive and seronegative subjects for the three main immunoglobulin classes. Comparisons were made by two-sample t-test and significant differences are denoted by an asterisk.

	P. gingivalis				*A. actinomycetemcomitans*			
	seropositive subjects		seronegative subjects		seropositive subjects		seronegative subjects	
	Mean PD (SD)	% BOP (SD)	Mean PD (SD)	% BOP (SD)	Mean PD (SD)	% BOP (SD)	Mean PD (SD)	% BOP (SD)
IgG	5.5 (0.8)	87 (19)	5.4 (0.6)	77 (21)	6.2* (0.5)	87 (21)	5.1* (0.4)	81 (22)
IgM	5.8 (0.9)	80 (21)	5.3 (0.7)	96 (7)	5.7 (0.6)	87 (14)	5.2 (0.7)	79 (14)
IgA	5.5 (0.2)	86 (18)	5.3 (0.8)	77 (23)	5.7 (0.8)	85 (21)	5.4 (0.7)	75 (12)

Table 28: Analysis of response to treatment according to initial serostatus in terms of IgG, IgM and IgA antibody to *P. gingivalis*. The average percentage of sites (SD) with PD >3.5mm and BOP at the end of HPT and at the first maintenance visit are given. Comparisons between seropositive and seronegative subgroups were made using two-sample t-tests.

		post-HPT % sites (SD)			Maintenance visit % sites (SD)		
		n	PD>3.5mm	BOP	n	PD>3.5mm	BOP
IgG	sero-positive	9	52 (18)	40 (20)	9	28 (23)	24 (16)
	sero-negative	8	73 (22)	39 (27)	7	43 (28)	45 (24)
	p value		0.047	0.958		0.288	0.082
IgA	sero-positive	11	55 (22)	42 (15)	10	28 (23)	23 (16)
	sero-negative	6	75 (16)	35 (34)	6	54 (29)	50 (21)
	p value		0.053	0.689		0.048	0.025
IgM	sero-positive	3	58 (44)	46 (7)	3	34 (31)	37 (37)
	sero-negative	14	63 (17)	38 (25)	13	30 (23)	32 (19)
	p value		0.872	0.337		0.317	0.834

Table 29: Analysis of response to treatment according to initial serostatus in terms of IgG, IgM and IgA antibody to *A. actinomycetemcomitans*. The average percentage of sites (SD) with PD >3.5mm and BOP at the end of HPT and at the first maintenance visit are given. Comparisons between seropositive and seronegative subgroups were made using two-sample t-tests.

		post HPT % sites (SD)			Maintenance visit % sites (SD)		
		n	PD>3.5mm	BOP	n	PD>3.5mm	BOP
IgG	sero-positive	5	66 (19)	39 (19)	5	44 (28)	26 (31)
	sero-negative	12	60 (24)	40 (25)	11	31 (25)	36 (17)
	p value		0.600	0.952		0.403	0.527
IgA	sero-positive	3	52 (13)	40 (24)	3	30 (18)	18 (16)
	sero-negative	14	64 (23)	39 (23)	13	36 (27)	37 (22)
	p value		0.278	0.971		0.662	0.154
IgM	sero-positive	8	70 (29)	45 (22)	7	43 (29)	33 (29)
	sero-negative	9	56 (13)	35 (24)	9	28 (21)	34 (16)
	p value		0.229	0.384		0.282	0.931

groups based on serostatus at baseline. The data for the response to *P. gingivalis* (Table 25), and *A. actinomycetemcomitans* (Table 26) are given. A large and statistically significant increase in avidity of IgG to *P. gingivalis* (0.69M, p=0.026) was detected only in the seropositive sub-group. By contrast, IgG and IgA titres to this organism tended to increase in the seronegative sub-group. However, Table 26 shows that there were no significant increases in antibody avidity to *A. actinomycetemcomitans*, but titres in all three classes increased significantly only in the seronegative sub-group.

An analysis of the differences between seropositive and seronegative patients at baseline in terms of clinical parameters was also performed (Table 27). This showed a tendency towards higher mean pocket depth and higher percentage of BOP sites in seropositive subjects. Since the inclusion criteria demanded that all sites should be >4mm at baseline, 100% of sites had PD > 3.5mm at this stage when related to later time-points in Tables 28 and 29. This table is presented to show that seropositive patients did not have better clinical presentation at

baseline which may have contributed to their better response to treatment.

The response to treatment was assessed after dichotomisation of the test group into subgroups according to serostatus at baseline. It should be noted that one patient dropped out of the study between post-HPT and the first maintenance visit. Table 28 presents the data according to serostatus for *P. gingivalis* and Table 29 for *A. actinomycetemcomitans*. Seropositive subjects for IgG against *P. gingivalis* demonstrated a significantly lower percentage of sites with PD > 3.5mm at the end of hygiene phase therapy. Similarly, seropositive subjects for IgA against *P. gingivalis* demonstrated a tendency towards lower % of sites with PD > 3.5mm at post-HPT and significantly lower % of deep and bleeding sites at the first maintenance visit. No significant differences in the response to treatment were observed between seropositive and seronegative subjects in terms of antibodies to *A. actinomycetemcomitans*.

The PCR analysis showed 5/17 (29%) of the patients being *P. gingivalis*-positive and 8/17 (47%) being *A.* actinomycetemcomitans-positive in at least one site at baseline.

Table 30: Differences between patient groups in terms of avidity of antibody to *P. gingivalis* in terms of median avidity (M) and median titre (EU) with inter-quartile range (Q1-Q3). * denotes significant difference at p=0.003.^ denotes significant difference at p=0.008.

	Mean avidity	Mean Titre
group 1	0.71 (0.27-1.09) *	6348 (1020- >10^5)
group 2	0.08 (0.02-0.37) * ^	1476 (415- 80508)
group 3	0.60 (0.16-0.96) ^	5666 (1142- 37021)

Table 31: Differences between patient groups in terms of avidity of antibody to *P. gingivalis* ARG-1 in terms of median avidity (M) and median titre (EU) with inter-quartile range (Q1-Q3).

	Mean avidity	Mean Titre
group 1	0.43 (0.21-0.72)	580 (118-2560)
group 2	0.49 (0.38-0.70)	1195 (54-3336)
group 3	0.50 (0.38-0.86)	1337 (59-5017)

3.3.3 Avidity of antibody to Arg-1 protease of *P. gingivalis* in relation to treatment effects

Forty three rapidly progressive periodontitis patients were used to carry out this study as a cross-sectional examination. The samples were divided into groups depending upon their treatment status i.e. untreated patients (Group 1), patients who were undergoing treatment but had shown no sign of improvement (Group 2-maintenance), and successfully treated patients (Group 3). Tables 30 shows a significant difference between group 2 and group 3 patients in terms of antibody avidity to *P. gingivalis* whole cells. However, this was not found for antibody avidity to *P. gingivalis* ARG-1 protease (Table 31).

Table 32: IgG subclass levels in non pregnant and normal pregnant women.

IgG Subclass	Non	Normal Pregnancy			
mg/ml	Preg	1st	2nd trimester	3rd	6week post
IgG1	1.7±0.9	3.1±0.8*	2.7±0.5+	1.6±0.4+++	1.6±0.9
IgG2	1.4±0.9	5.7±3.7***	2.0±0.9++	1.1±0.7+++	4.2±2.1 **
IgG3	0.4±0.1	0.8±0.1**	0.4±0.1++	0.5±0.2+	0.6±0.1
IgG4	0.6±0.2	0.5±10.1	0.4±0.2	0.2±0.1	0.4±0.1 *
Total IgG	4.0±2.0	10.4±4.2**	6.1±1.3+++	3.6±1.0+++	6.7±3.4

Data expressed as mean ± SD.

*P<0.03: **P<0.02: ***P<0.001 compared to Non pregnant

+P<0.02: ++P<0.04: +++P<0.001 compared to 1st trimester

Table 33: The distribution of IgG subclasses throughout the course of pregnancy and at 6weeks post partum in Group 3 patients.

IgG subclass (mg/ml)	1st trimester	2nd trimester	3rd trimester	6weeks post
IgG1	3.9±1.1	2.6±0.4*	1.9±0.6**	3.7±2.0
IgG2	5.4±0.5	2.6±1.1*	1.3±1.0**	2.0±1.0*
IgG3	0.7±0.1	0.6±0.1	0.5±0.1	0.4±0.2
IgG4	0.1±0.1	0.9±0.4*	0.3±0.1	0.7±0.3
Total IgG	10.6±0.5	6.8±1.9*	3.7±1.9**	7.2±2.8*

*p<0.03, **p<0.02 compared with 1st trimester.

3.4 Immunological parameters with obstetric significance

3.4.1 IgG1-4 in recurrent spontaneous abortion and successful pregnancy

The levels of IgG subclasses were measured by ELISA. Their levels in non-pregnant women are shown in Table 32. IgG1 was the predominant IgG subclass in non pregnant women. The serum concentrations of IgG1, IgG2, IgG3 and total IgG were significantly increased in the first trimester of a normal pregnancy. The rise in IgG2 was particularly significant (5.7±3.7 vs 1.4±0.9mg/ml), and this became the predominant subclass at 56.7% of the total IgG. In the second and third trimesters of a normal pregnancy, the serum IgG subclasses decreased gradually. At no time in the pregnancy was there any significant change in IgG4 levels. By 6 weeks post partum all values were returning towards normal.

The distribution of IgG subclasses in Group 3 women is given in Table 33. The results showed there to be no significant difference in IgG subclass distribution

Table 34: First trimester IgG subclass distribution in patients from Groups 3,4 and 5.

IgG SUBCLASS	GROUP2	GROUP 3	GROUP4	GROUP5
IgG1	3.1±0.8	3.9±1.1	2.4±0.4 ***++	2.3±0.7***
IgG2	5.7±3.7	5.4±0.5	3.2±0.9*	2.3±1.1*
IgG3	0.8±0.1	0.7±0.1	0.5±0.1**	0.7±0.4
IgG4	0.5±0.1	0.1±0.1	0.3±0.2	0.2±0.1
Total IgG	10.4±4.2	10.6±0.5	6.8±4.2*+	7.6±1.8*

All data is expressed as mean ± SD

*P<0.04; **P<0.004; ***P<0.002 compared to Group 2

+P<0.05; ++P<0.001 compared to Group 3

between Groups 2 and 3. The effects of miscarriage (either spontaneous or recurrent) on IgG subclass distribution are given in Table 34. Women with a history of RM, whose pregnancy again ended in miscarriage later on in the first trimester, was associated with significant decreases in levels of IgG1, IgG2, IgG3 and total IgG. Spontaneous abortion was associated with significant decreases in the levels of IgG1, IgG2 and total IgG. There were no significant differences between Groups 4 and 5. Early in the first trimester before the outcome of the pregnancy was known there were differences in levels of IgG1 between RA whose pregnancies continued and those whose pregnancies failed. In the small numbers studied an IgG1 level of less than 2.5mg/ml appeared to indicate miscarriage.

Table 35: Comparison between group 1 (RMc) and group 2 (RMa) in terms of median auto-antibody titre and avidity (interquartile range in parentheses) to TPO during first trimester.

	Group 1	Group 2	p-value
titre	3.2 (1.6-7.0)	66 (1.6-102)	0.1052
avidity	0.10 (0.06-0.15)	0.32 (0.24-0.44)	0.0015

3.4.2 Thyroid auto-antibody as a pregnancy outcome marker

Table 35 shows the comparison between RMc patients, i.e. those who had previously suffered from recurrent abortion but on this occasion continued to term (group 1), and RMa patients, i.e. recurrent abortion patients who again aborted during the first trimester (group 2), in terms of titre and avidity of auto-antibody to TPO. Median values are given with interquartile ranges and p-values for the comparisons between the two groups by Mann-Whitney test. Group 2 patients were found to have significantly higher titre and avidity of auto-antibody than group 1 patients.

3.5 Can fragments of bacterial DNA be detected in the host bloodstream?

No positive PCR reactions were detected for any of twelve sera tested.

Chapter 4

Discussion

4. Discussion

4.1 Local antibody studies

4.1.1 Humoral immunity in sites with differing clinical indices within the same patient

Specific IgG levels to *P. gingivalis* in GCF were found to be lower in periodontitis sites than in gingivitis sites in the same patient. Moreover, when correction is made for the systemic contribution by expressing results as EU/30s sample/mg albumin, this distinction appears more significant suggesting that increased consumption of locally-produced antibody in periodontitis sites may be responsible for this difference. Alternatively, there may be a failure of local production, or adsorption by plaque bacteria, or a combination of these mechanisms. Lamster et al (334) reported a significant correlation between total IgG in gingival crevicular fluid and specific serum antibody to *Bacteroides intermedius* but not *P. gingivalis*. They conclude that the development of a serum immunoglobulin response to suspected periodonto-pathogens is consistent with a protective host response. A corollary of this view is that a local deficiency of IgG

to *P. gingivalis* may lead to local disease progression. Alternatively, Kilian (145) has demonstrated that *P. gingivalis* can degrade human IgG and IgA, suggesting that low gingival crevicular fluid levels of IgG may be caused by degradation by *P. gingivalis*, or that locally available antibodies are adsorbed by the greater mass of subgingival plaque present.

Baranowska et al (331) found no difference between levels of specific IgG to *P. gingivalis* in healthy and diseased sites within the same individual. However, fixed volume samples of 0.5μl were taken. This means that strips from sites which did not yield 0.5μl were discarded and that samples were taken over variable time periods resulting in variable rates of serum contamination (398). Since healthy sites tend to yield lower volumes than diseased sites, this method treats healthy and diseased sites in different ways. Therefore, these results are not strictly comparable with the present study. An earlier study by Schenck (335) showed an inverse relationship between the number of deep pockets (>4mm) and serum antibody level to *P. gingivalis* lipopolysaccharide in chronic periodontitis patients. Mouton et al (336) demonstrated a dichotomy in serological responses to *P.*

gingivalis among chronic periodontitis patients with one sub-group exhibiting high serum antibody levels and another having levels similar to those of healthy individuals. The patients with high serum antibody levels appeared to respond better to therapy as evidenced by reduction in the number of deep pockets within one month post-treatment. These findings are consistent with the data reported here that periodontitis sites have lower IgG levels to *P. gingivalis* than gingivitis sites in the same patient.

It has been shown that GCF IgG antibody can inhibit colonisation of the gingival root surface by mutans streptococci (399). Bacterial immunoglobulin cleavage activity in GCF has been shown to have a rôle in localised juvenile periodontitis (400).

It should also be noted, however, that a recent paper by Gemmell et al (274) has shown that adult periodontitis patients tend to produce serum antibodies to *Porphyromonas gingivalis* lipopolysaccharide (LPS) to a much greater extent, and to higher molecular weight antigens to a much lesser extent, than gingivitis subjects. Highlighting the indications that LPS does not induce a protective response, their results suggest that periodontitis

pathology may be related to an inability to target high molecular weight antigens, e.g.. the 91.4kDa band. In the present study it may be that the whole cell preparation used in the assay allows detection of antibodies to high molecular weight antigens to a greater extent than antibodies to LPS, thus contributing to the observed difference between periodontitis and gingivitis sites.

The present findings confirm previous observations in this laboratory (338) and those of OuYang (339-340). The indications of these studies are that a failure of local antibody production or reduced amounts due to e.g. degradation by bacterial proteases, may contribute to the switch from gingivitis to periodontitis pathology. The present study has corrected for systemic factors by performing paired analysis within the same patient and allowed for systemic input by the use of albumin assays. This permits the tendency of reduced IgG to *Porphyromonas gingivalis* in periodontitis sites to be more clearly seen. Moreover, this appears to be the first report to indicate an immunological difference between gingivitis and periodontitis sites purely on the basis of clinically-assessed site disease status. However, a larger study comprising both a larger patient group and a greater

number of sites sampled per patient is necessary to corroborate these findings.

4.1.2 Effects of ageing on immune and inflammatory responses during experimental gingivitis

The results of the present study suggest that there are differences between young and old periodontally-healthy individuals in terms of immune and inflammatory responses during the course of experimental gingivitis. In particular, IgG1 and IgG4 levels in GCF are lower in old subjects and IgG3 is higher than in young individuals. Analysis of levels as a ratio to albumin suggest greater local synthesis of IgG3 and lower synthesis of IgG1 in older subjects. Moreover, LF levels were lower in older individuals suggesting reduced PMN infiltration, since it has previously been shown in this laboratory that the level of this protein is correlated with the number of crevicular PMNs (401). The ability of these acute-phase proteins in GCF to distinguish healthy, gingivitis and periodontitis sites has also been demonstrated in this laboratory (377).

Ebersole and Cappelli have demonstrated that >95% of sites with elevated IgG4 antibody to *Actinobacillus actinomycetemcomitans* were colonised with the organism,

whereas <50% of sites with elevated IgG2 demonstrated this microorganism (402). They conclude that the frequency and distribution of antibody in the GCF as related to colonisation with this microorganism were consistent with localised host-parasite interactions at the individual tooth sites, and suggest that the potential exists for this local antibody to play an important role in the gingival sulcus in relation to colonisation and clinical presentation. Moreover, it has also recently been demonstrated in this laboratory that strong correlations exist between IgG subclass levels in GCF and in serum and IgG1-4 mRNA expressing plasma cells in the gingiva (403).

It is generally accepted that there are changes in the humoral immune response with ageing. For example, a decreased response to vaccination and defective specific antibody synthesis have been demonstrated in ageing humans (212,213).

Interestingly, Hu et al found that old mice had an increased CD5+ B-cell response but decreased CD5- response. This is related to the increased ability of aged animals and humans to produce autoantibodies whilst having an impaired response to foreign antigens (404). However, in terms of the specific antibody response to

plaque antigens in experimental gingivitis, Lekic found that this was increased in adult rats as opposed to young rats (405). It should be borne in mind that this study was in a rat model and the distinction was between adult and juvenile rather than elderly and young individuals.

Additionally, Spector et al have demonstrated that psychological conditioning of the immune response may be an important factor in ageing (406). Bovbjerg et al report that dysregulation rather than exhaustion of the immune response is the most important factor with ageing, i.e. there is an increased autoantibody response despite a reduced response to foreign antigen (407).

It has been shown here that there are differences between the immune and inflammatory responses in young and old individuals during the course of experimental gingivitis. These age-related differences may have a bearing on the natural progression of periodontal disease with ageing in humans. However, further studies are required to establish whether these mechanisms are important during the normal course of periodontal disease.

4.1.3 Humoral immune response at matched tooth and implant sites in the same patient

Osseointegrated implants represent a realistic alternative treatment for partial and total edentulism. This long-term success has been established in longitudinal studies to be well over 90% after ten years. Once osseointegration has been achieved, such high success rates depend upon the establishment and maintenance over time of a host-parasite equilibrium in the peri-implant sulcus.

Recent studies have determined that peri-implant microbiota (408-411) and peri-implant anatomy and function (412) are generally comparable to those pertaining to periodontal tissues.

The dearth of studies dealing with the local humoral immune response in PISF prompted us to conduct the present study. Tables 9-12 show that specific antibody titres tended to be lower and measures of local production, i.e. RCE ratios, tended to higher in implant sites than in matched natural tooth sites. These differences were not statistically significant, but they

may provide an indication that specific antibody levels are lower around implants and also that proportionately more of that antibody is locally produced.

This may suggest differences in T-cell traffic to the gingiva between the periodontal and peri-implant environment. Naive and memory T-lymphocytes demonstrate different traffic patterns (413). The effect of antigenic stimulation is clonal expansion and differentiation of lymphocytes from naive into effector or memory cells. Memory lymphocytes acquire the ability to migrate into tertiary lymphoid tissues. Once activated, lymphocytes are not relatively homogeneous like naive cells but show selectivity in homing behaviour (414,415). Some studies have indicated that memory or activated lymphocytes traffic preferentially back to the tissue where they were first activated. Selective homing of memory lymphocytes can be mediated by differential regulation of receptors, which are adhesion molecules needed to recognise and bind to endothelial cells within different tissue sites.

Those memory T-lymphocytes can selectively home to non-lymphoid (or tertiary lymphoid) tissues (416,417). Therefore, most T-cells found in tertiary lymphoid

tissues (such as gingiva) are memory cells (418). Binding to endothelium (419) seems to be the first step in the transmigration of lymphocytes into the tissues (420). This is regulated by the differential expression of surface molecules on the effector lymphocyte and also on the endothelium of the target tissue. Lymphocyte homing is therefore initiated by surface molecule expression.

Therefore, it may be that the observed differences between periodontal and peri-implant antibody levels reflect differences in lymphocyte traffic into these two environments. A larger study is required to establish whether or not this is the case.

Tables 13-15 demonstrate that the correlations between immunoglobulin class responses to specific organisms which pertain for natural teeth do not hold for matched implants, and also that rates of local antibody production of the IgG and IgM classes do not correlate for matched teeth and implants. These observations indicate that class switching (e.g. IgM->IgG) in peri-implant tissue may also be interfered with by some mechanism involving changes in the micro-environment.

These findings suggest differences in the plasma cell infiltrate in natural teeth and implants. Further studies focusing on GCF and PISF antibody levels in conjunction with comparative studies of cellular events at matched teeth and implants are required to elucidate these findings.

4.1.4 Checkerboard immunoblotting of GCF

The pilot study carried out here suggests that this technique has the potential to allow multiple assessments of antibody reactivities with antigen. However, since GCF samples had to be diluted hundredsfold before analysis, low antibody concentrations may not be picked up. Moreover, the results yielded are qualitative, making it difficult to make definite statements about relative levels in different types of sites. Further studies would involve quantitation using laser densitometry provided that the membrane substrate can be rendered transparent.

4.2 *In situ* hybridisation studies

4.2.1 Study of κ/λ light chain producing plasma cells in gingival tissue

There is a complex involvement of the cellular and humoral immune responses in the pathogenesis of periodontal disease. It is generally accepted that B cells and plasma cells are abundant in gingival tissues affected with moderate and advanced periodontitis and these cells locally produce immunoglobulin G (IgG), IgA and few IgM positive cells as seen by immunohistochemical techniques (421-424). The immunohistochemical staining for immunoglobulin using antibodies are however often unreliable due to the high background staining that may be derived from non-specific binding of immunoglobulins in the tissues (425) and serum derived immunoglobulin (426).

In addition, the detection of immunoglobulin protein *in vivo* presents several technical difficulties because

the protein can be degenerated by neutrophil elastase (427) and oxidant (428) in inflamed sites. Moreover, it has been reported that periodontal bacteria secret IgA1 proteases which cause the cleavage in the hinge region resulting in Fab and Fc fragments (146). Wilton *et al.* have recently reported that IgG subclasses, except IgG_4, in gingival crevicular fluid (GCF), are largely derived from plasma. They based this conclusion on the poor correlation between the amounts of IgG subclass proteins in GCF detected by ELISA and the clinical indices (429). Controversies exist regarding the relative importance of local and systemic antibody synthesis in the periodontium.

Immunolocalisation of immunoglobulins at the protein level presents many technical difficulties and also difficulties in interpretation. *In situ* hybridisation is a molecular biological technique which permits the detection of specific messenger RNA (mRNA) expression even in small tissue samples. This technique avoids many of the problems alluded to above and offers the opportunity to detect, localise and quantify the cells producing immunoglobulins. It has been reported that the *in situ* hybridisation method for detection of immunoglobulin light chain mRNA positive cells may give different results to

those obtained by immunohistochemistry (425,430,431). Further, although the ratio of κ to λ light chain positive cells varies in different sites and diseases (432-434), no data exist for the expression of immunoglobulin light chain mRNA in inflamed gingival tissues from patients with periodontitis. This study therefore investigated the localisation and proportions of immunoglobulin light chain mRNA positive cells in inflamed gingiva using the *in situ* hybridisation (ISH) technique and considers their potential relevance to the local humoral immune response of the periodontium.

This study confirms that immunoglobulins are locally produced by plasma cells within the gingival tissues of patients with periodontitis and further indicates that the predominant plasma cells are κ light chain producing cells. Although immunoglobulin in the GCF of patients with periodontitis may be derived from the systemic circulation (426,429), it is likely that the most relevant immunoglobulins are produced by local plasma cells at these sites. The build-up of these "periodontal disease relevant" plasma cells may arise via selective homing of specific lymphocytes and/or by the clonal proliferation of specific B cells in this region (368,435).

Immunoglobulin light chain mRNA positive cells were clearly seen in all sections. The *in situ* hybridisation method performed in this study has particular advantage over immunohistochemistry in studies where the gene products are rapidly exported from the cell into the tissue and systemic circulating immunoglobulin may have influence. Previous efforts at immunolocalisation of immunoglobulin using monoclonal antibody techniques in this laboratory have been disappointing due to the excessive background staining thought to be due to the systemic and local soluble immunoglobulin presence. Further, it has been reported that the cytoplasmic immunoglobulin detected by immunohistochemical methods may not be a result of synthesis but of uptake by the cell (425,431). *In situ* hybridisation methods for detection of immunoglobulin light chain mRNA positive cells may give different results to those obtained by immunohistochemistry (425,430,431) which would explain why the results presented here differ from the previous report employing immunohistochemistry (436). In these circumstances, the *in situ* hybridisation technique shows precise localisation of gene expression free from the

problems associated with the detection of extracellular targets and their possible uptake by other cells.

This study demonstrates a predominance of κ light chain positive cells (κ : λ = 2.4 : 1) among plasma cells in the human inflamed gingival tissues. Low κ/λ ratios have been reported for other inflamed tissues such as duodenum, tonsil (432) and renal biopsy samples from patients with IgA nephropathy (433). Moreover, an increase in IgG-1 has been correlated with infectious status (434) although the precise reasons are unknown. These reports suggest that the ratio of the two immunoglobulin light chains may vary due to the severity of the lesions such that there may be heterogeneous responses even in related inflammatory diseases. The κ/λ ratio of inflamed sites could be a useful method of distinguishing the stage or activity of periodontitis lesions and may differ between different forms of periodontitis.

4.2.2 IgG and IgA subclass production by plasma cells in periodontal tissue

This is the first report demonstrating IgG and IgA subclass-specific mRNA-expressing plasma cells in inflamed gingival tissues affected with periodontitis. The results also suggest the possibility that class switching pathways are induced in periodontitis sites and these may provide a basis for understanding the mechanisms of B cell differentiation in these sites, although it cannot be ruled out that the switching occurs in local lymph nodes; committed B cells home to gingival tissues, and periodontally diseased tissue may have lymph node-like features.

This is also the first report of the use of oligonucleotide probes for IgG and IgA subclass expression studies, although an earlier study used RNA probes for IgA (437). Oligonucleotide probes, because of their size, are more able to penetrate permeabilised tissue sections; allow for less cross-reactivity than that seen with full-length cDNA probes, and hybridize in the presence of formamide with retention of good tissue morphology. In

addition, oligonucleotide probes are much easier to work with than RNA probes, due to their greater stability. In this study digoxigenin labeled oligonucleotide probes were used allowing the completion of this *in situ* hybridization procedure within two days, obtaining very high contrast and optimal histological resolution. The disadvantage of this non-isotopic *in situ* hybridization technique may be lower sensitivity than the autographic method, but IgG and IgA subclass-specific mRNA could easily be detected in all gingival tissues tested. As a preliminary, the hybridisable mRNAs in the tissue sections were examined by using poly d(T) oligonucleotide probes. Plasma cells and epithelium, especially basal layers, were usually strongly stained. Furthermore, strong signals for Ig light chain mRNA in plasma cells were shown by non-isotopic *in situ* hybridization (438).

It is important to note that optimization of proteinase K digestion was the most important step in this work in terms of enhancing signal strength and a narrow optimum for proteinase K digestion exists for maximum hybridization without significant destruction of cytological structure and solubilisation of the target sequences. The effects of varying the proteinase K

concentration on the *in situ* hybridization signal has been previously demonstrated (430).

IgG1 mRNA-expressing plasma cells were the major subclass of IgG mRNA bearing plasma cell detected, which is similar to previous literature (439), and levels reported for normal tissues (440) and synovium from patients with rheumatoid arthritis (441). In contrast, a relative increase in IgG4 plasma cells was shown in this study and it is similar to previous reports (439,429). The subclass distribution of IgG antibodies produced as a consequence of bacterial infection varies according to the nature of the eliciting antigens. Protein antigens induce mainly IgG1 antibodies, with small amounts of IgG3 and IgG4 antibodies (442,443). In contrast, IgG antibodies reactive to carbohydrate antigens, including lipopolysaccharide (LPS), are predominantly IgG2. Since certain antigens elicit an Ig response largely confined to one IgG subclass (444,445), the distribution of IgG and IgA subclasses in gingiva may provide clues to pathogenic mechanisms.

Protein antigens from periodontal bacteria or their products may induce one subset of B cells to switch subclasses that are clustered in the 5' region of the Ig

heavy chain gene. Another switching pathway of Ig constant regions may occur in the 3' region (g2-g4-e-a2-3') which seem to be predominantly involved in LPS-specific responses. It has been reported that the protein and lipid carbohydrate antigens of *P. gingivalis* induce two distinct patterns of IgG and IgA subclass responses in inflamed gingiva (446). Therefore it is possible that the change in IgG and IgA subclass obtained is due to the change of microbial antigens or possible other mitogens involved at various stages of periodontitis. The data presented here shows reduced local production of IgG2 which suggests that the response to carbohydrate antigens may be less important at the local level than protein antigens.

This is supported by the observation that local IgG4 production is much higher than systemic levels would indicate as normal. However, actual levels of IgG4 and IgG3 are much lower than indicated by their local production levels (specific plasma cell levels), but these findings must not be interpreted too strictly as much variation exists, particularly with these subclasses which are present in low percentages of total IgG. Several studies indicate that the average serum proportion of the

IgG subclasses is as follows: IgG1 65%, IgG2 30%, IgG3 5% and IgG4 4% (378). IgG1 appears at constant levels in terms of systemic and local concentration and local production. However, IgG2 local production is reduced relative to local and typical systemic levels, which could suggest, as IgG2 is linked with Ig responses to carbohydrate antigens, a reduced involvement of the local response to carbohydrate antigens in the periodontium. The local levels of IgG3 and IgG4 are substantially reduced relative to their serum concentration and levels of local production but these immunoglobulins are at low numbers which makes analysis and thereby inferences less reliable.

In terms of IgA, predominance of IgA1-producing cells (65.1%) was observed, however, IgA1-producing cells were detected in equal proportions to IgA2 in 5 out of 24 sections (2 sites were from same subject and the others were from different subjects). The gingival tissues are exposed to a heavy bacterial load, and IgA2 is resistant to several bacterial proteases which can cleave IgA1 (146). Therefore, increased IgA2 production would appear to be advantageous in the periodontitis gingiva and should aid killing of bacteria in the GCF. However, these

results are not in accordance with the previous reports on the percentage of IgA1-producing cells determined by immunofluorescence (447) or enzyme-linked immuno spot (ELISPOT) assay (439) which have reported proportions of more than 90%, similar to those seen in serum (448). Immunohistochemical studies for Ig in inflamed sites are often unreliable due to high background staining, as has been established in this laboratory. In an ELISPOT assay described by Ogawa *et al.* (439,446), gingival mononuclear cells were isolated and cultured for 3h before the assay, suggesting no effect of IgA1 proteases for IgA1 production in their *in vitro* system. Further, although the amount of IgA subclass mRNA expression in each cell could not be quantified, the rate of IgA1 subclass secretion may be higher than that of IgA2. Further investigation of these points would be worthwhile.

IgA antibodies specific for protein antigens are found predominantly in the IgA1 subclass, while specific antibodies to LPS and lipoteichoic acids are predominantly of the IgA2 subclass (449,450). Therefore the distribution of IgA1- and IgA2-producing cells in inflamed gingiva may reflect a difference in the predominant type and nature of antigens involved in the induction of the

local humoral immune response in periodontium. The observed preponderance of IgA1 or IgA2 cells in gingival tissues may be due to differences in the original precursors of IgA plasma cells destined for population of gingival tissue, selective homing of specific B cells and/or the local expansion of specific B cell clones by various antigens that induce preferential IgA1 or IgA2 responses (368,435).

B cell differentiation and the regulation of the switch to different Ig isotypes is cytokine driven (451, 452). TGF-β1 and/or IL-4 increase the level of a germ-line transcript, and then B cells are induced to switch from membrane-IgM^+ to membrane-IgA^+ (453). TGF-ß1 but not IL-4 mRNA was detected by the reverse transcription-polymerase chain reaction method in inflamed gingival tissues (454). Therefore TGF-β1 could influence IgA subclass switching in periodontitis gingiva, although TGF-β1 mRNA signals could not be detected by the *in situ* hybridization method described in this study. Recently it has been reported that germ-line transcripts are relevant to the Ig class and isotype switch recombination in the *in vivo* immune response in humans (455). In addition to the

influence of cytokines, direct cell to cell interaction may also be involved in isotype switching mechanisms. IgA subclass-specific mRNA bearing plasma cells were seen in sections as discrete cells, i.e. not clustered. IgA mRNA bearing plasma cells, however, were often noted in contact with IgG mRNA expressing plasma cells, as detected by double-target *in situ* hybridization.

IgG and IgA subclass proteins were also detected in the GCF. Because local cellular events in gingival tissue must always be related to the events in the GCF, i.e. the quantities and specificities of antibody proteins actually being released and deployed locally. There was no correlation between the amounts of IgA subclass protein in GCF and the numbers of IgA mRNA-positive cells of the same subclass at the same sites, although strong correlation between the amounts of IgG subclass protein in GCF and the numbers of IgG mRNA-positive cells. These results suggest that IgG in GCF is composed of serum derived and locally produced IgG subclasses, although determining the actual ratio of serum derived to locally produced will be technically complex and thus this ratio is presently unknown. It has been reported that Ig can pass from serum to the oral cavity via GCF (426) and that large

proportions of the IgG in GCF is derived from plasma (429). A possibility is that serum IgA subclasses influence the GCF proportions. This is unlikely however, because serum IgA1 is always approximately 90% of total IgA (448), whereas the proportions of IgG in GCF were similar to that of serum making this hypothesis unlikely.

Higher concentrations of IgA1 Fab fragments than intact IgA1 were detected. It has been reported that periodontal bacteria degrade IgA1 (145). Several explanations to this discrepancy are possible. First, the monoclonal antibody used reacted strongly with Fab fragments of IgA1 produced by specific IgA1 proteases from *Prevotella* and *Capnocytophaga* species and weakly with Fab fragments induced by specific IgA1 proteases of other cleavage specificities. Therefore, a possible explanation for this lack of correlation between IgA1 producing cells and IgA1 concentration in GCF may be that IgA1 was degraded by periodontal pathogen-derived IgA1 proteases. Another possibility is that IgA1 may bind to bacteria and migrated PMN in GCF both specifically and non-specifically rather than IgA2. Thirdly, IgA subclass-positive cells at the mRNA level were investigated, but further mechanisms are involved in the translation stage i.e. prior to Ig

being released, and thus the possibility exists that although IgA1 mRNA positive cells are abundantly present their protein products may not be as readily released as say IgA2.

4.3 Antibody avidity studies

4.3.1 Preliminary study of treatment effects in refractory patients

This study presents findings from a small group of patients that appear to be resistant to periodontal treatment. The compliance of these patients with their respective treatment plan had been good and their oral hygiene was always within acceptable limits (386). Apart from the presence of putative periodontal pathogens in the gingival crevice of all patients, perhaps the strongest common factor to the continuing disease progression in eight of these subjects was that they smoked. Cooperative patients that nevertheless fail to respond to periodontal treatment are recognised in most clinical periodontal practices (456-457), although fortunately they form only a small proportion of patients.

The presence of putative periodontal pathogens in the test sites in these patients from baseline to week 52 has been described elsewhere (386). Elimination of *P.*

gingivalis was achieved more readily than for *A. actinomycetemcomitans* in most cases. The potent antibiotic combination chosen in this study has been shown to be effective in eliminating *A. actinomycetemcomitans* from the periodontium in patients with localised juvenile- and rapidly progressive periodontitis (458). In the present study, however, this antibiotic combination failed to eliminate *A. actinomycetemcomitans* in all of these subjects by the end of the study at week 75 although *P. gingivalis* could not be isolated from any test site by then. The success of most antibiotics in eliminating even sensitive organisms is, to a greater or lesser degree, dependent on assistance from the patient's immune system. A failure of the immune system at the local level in these patients with resistant periodontitis may explain why the antibiotic was not able to eliminate the organism.

The present study confirms previous findings that antibody avidities to suspected periodontopathogens are higher in patients with periodontal disease than in age/sex matched control individuals (330). Specifically, this investigation has shown that

avidities of antibodies in all three immunoglobulin classes to *P. gingivalis* and IgG to *A. actinomycetemcomitans* are higher than in controls. Chen *et al,* (273) found that RPP patients had low avidities that subsequently rose after treatment. This was not found in the material of the present study perhaps because these patients had been on regular recall for hygiene therapy before the baseline measurements were made (386). The longitudinal element of the study, however, failed to show any significant differences in antibody avidity during the course of treatment. Previously, it has been shown that adult periodontitis patients who were initially seropositive developed higher avidity antibodies to *P. gingivalis* during the course of therapy and had a better response to treatment (459). The present group of ten patients was not suitable for this type of analysis. For example, seven out of the ten patients were seropositive for *P. gingivalis* at baseline, making sub-grouping impossible. More importantly, the present study was of patients who had proved to be resistant to treatment, and these may well be an immunologically distinct group.

There have been a number of reports suggesting that there is a relationship between immunological parameters and treatment response in adult periodontitis and early-onset periodontitis. A recent report by Ebersole *et al*, (460) quotes data showing that 60% of periodontitis patients exhibited increased titres to a battery of suspected periodontopathogens post-treatment, and that these patients had fewer episodes of active disease during the monitoring interval (two years) than those who did not demonstrate increased titres. Mouton *et al*, (336) demonstrated that initially seropositive patients responded better to therapy than those who were initially seronegative. Moreover, the tendency to develop further attachment loss in maintenance patients is related to antibody avidity to *P. gingivalis* (330).

Gingival crevicular fluid (GCF) IgG titres to *P. gingivalis* have been found to be lower in deeper pockets and more inflamed sites in maintenance patients (338). This suggested that failure of production of antibody at systemic and/or local level combined with failure of deployment at local level, perhaps caused by low binding strength (avidity) and/or degradation or inactivation of immunoglobulin by microorganisms (145-

146), may lead to further periodontal breakdown on a site-specific basis.

The clinical, microbiological and immunological findings in this small group of well-motivated patients suggest that continued disease progression in patients resistant to periodontal treatment may be related to a defect or deficiency in their immune system. With the exception of IgA titres to *P. gingivalis* antibody titres were not significantly higher in these patients than controls. Avidities were significantly higher for each class of antibody to *P. gingivalis* but only for IgA to *A. actinomycetemcomitans*. Clearly these patients mount an immune response to these putative periodontal pathogens but it does not seem to be adequate to stop disease progression. The underlying immune defect, if such exists, presumably is at a very local level. Further studies of this type of subject may help to elucidate the balance between host factors and putative pathogens in adult periodontal disease. A larger study group is required but, as this type of patient is not common, a multi-centre collaborative investigation seems worthwhile.

4.3.2 Longitudinal study of immunological parameters in relation to treatment outcome

The results presented here suggest that the further development of the humoral immune response to suspected periodontopathogens is predicated on serostatus before treatment commences. For example, Table 25 demonstrates a large and statistically significant increase in IgG avidity to *P. gingivalis* for initially seropositive patients, whereas seronegative patients showed no change in avidity but a tendency towards increased titres (+474EU, p=0.08) which failed to reach statistical significance. Initial serostatus is dependent on a number of factors including timing and extent of previous exposure to the subgingival microflora, the nature of the latter and host susceptibility.

The effect of treatment on antibody responsiveness may result from an inoculation effect during scaling and root planing, or from the reduction in antigen load resulting from these procedures and improved oral hygiene, or from a combination of these two mechanisms. Reduction in antigen load is known to result in selection of B-cell

clones producing higher avidity antibodies (182). Treatment may also permit the development of a normal maturation of the immune response, perhaps mediated by an inoculation effect, leading to antibodies of increasingly higher avidity.

Patient sub-grouping was performed by Chen at al (273) in their investigation of treatment effects on rapidly progressive periodontitis patients. They found that avidities of IgG antibody to *P. gingivalis* increased in their seropositive sub-group after therapy, whereas titres decreased. Therefore, the present study tends to confirm this effect in adult periodontitis patients. It should also be noted that antibody avidities to periodontopathogens are generally low in humans compared with those to other commonly occurring antigens, and also when compared with avidities observed in animals immunised with periodontopathogens (273,190,325).

The present study, being concerned with the systemic immune response, did not set out to relate site microbiology to site or systemic immune response. However, plaque samples were analysed by PCR in order to provide some data on this point. Mombelli et al (461) showed that, when whole-mouth analysis for *P. gingivalis*

was carried out in periodontal disease patients, 30% of patients were organism-negative in all sites. Moreover, a further 30% of patients had this organism in only 1-10% of sites. This suggests that, at any particular time, a maximum of 40% of patients with periodontal disease would be classified as *P. gingivalis*-positive by a representative sample of 8 sites. Our PCR analysis showed 29% of patients being *P. gingivalis*-positive and 47% being *A. actinomycetemcomitans*-positive. This compares well with another recent study in our laboratory showing 12/43 (28%) patients being *P. gingivalis*-positive and 40% being *A. actinomycetemcomitans*-positive based upon representative site sampling (462).

Little data exists on the relevance of differences in immune response to periodontopathogens to clinical improvement. However, Mouton et al (336) demonstrated that initially seropositive patients responded better to therapy than those who were initially seronegative. Moreover, a recent study by our group suggested that the tendency to develop further attachment loss in maintenance patients is related to antibody avidity to *P. gingivalis* (330). In an earlier report from this laboratory, it was demonstrated that gingival crevicular fluid (GCF) IgG

titres to *P. gingivalis* were lower in deeper pockets and more inflamed sites in maintenance patients (338). This suggested that failure of production of antibody at systemic and/or local level combined with failure of deployment at local level, perhaps caused by low binding strength (avidity) and/or degradation or inactivation of immunoglobulin by microorganisms (146,396), may lead to further periodontal breakdown on a site-specific basis.

A recent experimental gingivitis study by Danielsen et al (463) showed that reductions in serum IgG titres to *P. gingivalis* during the experiment were predominantly attributable to subjects who had high initial titres and who went on to develop marked gingival inflammation during plaque accumulation. They suggested that the development of more extensive gingival inflammation in these subjects, perhaps equivalent to seropositive or previously sensitised individuals, could be a prerequisite for successful reduction or elimination of the organism through protective immune and inflammatory mechanisms.

Moreover, Ranney et al (464) and Gunsolley et al (345) demonstrated less disease in patients with higher antibody titres than in those with lower titres. However, a contradictory finding by Chen et al (273) was the

observation that there was a statistically significant, albeit weak, negative correlation between IgG antibody avidity to *P. gingivalis* whole cell sonicate and both severity of bone loss and pocket depth. This latter study, however, investigated RPP rather than adult periodontitis subjects and the negative correlation did not apply for *P. gingivalis* LPS or protein. The significance of this study is, therefore, difficult to assess.

The present study indicates that the characteristics of the humoral immune responses to *P. gingivalis* and *A. actinomycetemcomitans* serotype a may be quite different. Avidities to *P. gingivalis* tended to increase after therapy in seropositive patients, whereas titres to *A. actinomycetemcomitans* tended to increase in seronegative patients.

Table 24 demonstrates that the increase in IgG avidity to *P. gingivalis* during the course of therapy in the entire patient group is, when considered with Table 25, attributable to the influence of the seropositive sub-group. Our data suggest that seropositive subjects, in terms of IgG and IgA against *P. gingivalis*, demonstrate a tendency toward a higher chance of clinical improvement

after conventional periodontal therapy (Tables 28 and 29). This is despite the finding that seropositive subjects tended to have deeper mean pocket depths at baseline (Table 4). Although the significance of our findings is weak, our data agree with the observation of Mouton et al (336) who demonstrated that initially seropositive patients responded better to therapy than those who were initially seronegative. This observation would also concur with those of Ebersole et al (465) and Danielsen et al (463). The suggestion is that the prior development of a protective (although in an attenuated fashion) humoral immune response has a positive contribution to disease resolution during and after therapy. A recent study by Sjostrom et al has also indicated that scaling and root planing induces a humoral immune response which has a role in the beneficial effects of periodontal treatment (466).

There are indications that the humoral immune response to periodontopathogens may be unique in character. There have also been indications from previous studies and the present study that previous exposure and host susceptibility have a bearing both on the development of periodontal disease and response to treatment. However, further work on the effects of periodontal

treatment on the humoral immune response is required to elucidate this phenomenon.

4.3.3 Avidity of antibody to ARG-1 protease of *P. gingivalis*

The results presented in Tables 30-31 suggest that, although these outcome groups could be distinguished in terms of antibody avidity to *P. gingivalis* whole cells, this was not found for reactivity to the ARG-1 protease of this organism. This may suggest that other proteins are more important as virulence factors in RPP.

4.4 Significance of immunological parameters in pregnancy

As was touched upon in the Introduction (section 1.4.4.), Lopatin *et al* (190) have speculated that *in utero* exposure to periodontal antigens may occur because of pregnancy gingivitis, which is often severe leading to bacteraemia (214-216), followed by placental transfer. Clearly, if this occurs early during immune maturation, there is the real possibility that these antigens will be recognised as auto- rather than allo-antigens. Similarly, Collins et al have shown recently that *P. gingivalis* has deleterious effects on pregnancy outcome in hamsters (467-468). Significantly, this effect can also be detected with heat-killed cells and also LPS from this organism. These latter two studies suggest that bacterial antigens do cross the placenta.

Moreover, Nelson et al have demonstrated that a disparity in HLA class II alloantigens is associated with the remission of rheumatoid arthritis which has previously been described in pregnancy (469). A mechanism has been postulated involving maternal tolerance to foetal

antigens. This prompted a preliminary study of IgG subclass responses during pregnancy, since previous studies have linked the absence of an IgG blocking factor with foetal rejection (239-242). It was not possible within the present project to determine whether or not bacterial antigens penetrate into the maternal/foetal circulation or reactivity of the subclasses with specific bacteria, but this would appear a promising area for further research.

4.4.1 The relationship between serum levels of IgG subclasses and pregnancy outcome

Miscarriage is the most common complication affecting human gestation. It has been suggested that the rate of women who miscarry three consecutive pregnancies is 0.4% of all clinically recognised pregnancies (470-471). This pilot study is the first to detect the distribution and changes in IgG subclasses during the course of a normal pregnancy and those suffering recurrent miscarriage (RM). The data from this study has shown an association between levels of IgG subclasses and normal

pregnancy or pregnancy loss. It was found that the concentrations of serum IgG1, IgG2 and IgG3 were significantly increased in the first trimester of a normal pregnancy compared to non pregnant women. While IgG1, at 65% of total IgG, is the dominant IgG subclass in non pregnant women, IgG2 increased markedly and became the dominant IgG subclass in the first trimester of a normal pregnancy. During pregnancy, serum levels of IgG1, 2 and 3 decreased gradually, but returned towards non pregnant levels during the six weeks following delivery.

Previous studies have shown that IgG and IgM production was significantly raised in the first trimester of a normal pregnancy (244-245). It has been proposed that an increase in B cell numbers or B cell activity may account for the increased production, although from the studies carried out to date it is not clear which is responsible. There have been reports in the literature of increased numbers of B cells occurring in normal pregnancy (472).

An investigation was also conducted into why some women with a history of recurrent miscarriage should have a pregnancy that continued to term whilst others

miscarried again later on in the first trimester. Comparison of the IgG subclasses showed the levels of IgG1, IgG2 and IgG3 to be significantly lower in RM who again miscarried. Similar results were found in women admitted suffering spontaneous abortion. Why and how IgG production should be blocked in these women is not clear. In this small pilot study levels of IgG1 appeared to act as a marker for miscarriage in these women.

It is well known that the foetus inherits antigens from the father that are foreign to the mother. The mechanism by which the foetus evades immunological rejection is unknown. Early in the 1970's Rocklin and his co workers found that maternal lymphocytes can produce an immigration inhibitory factor in normal pregnancy (239). Their studies on the nature of the blocking factor indicated that this factor was an IgG antibody and it was directed against paternal antigens. Moreover, women who abort lack this antibody in their sera (240-242). The mechanism by which IgG exerts its influence is not yet completely understood. It has become clear that IgG has immunosuppressive properties (473). Several recent studies have suggested that during pregnancy IgG may block antibody binding to Fc receptors

of target cells, thus prohibiting local antibody effects. Feedback inhibition of antibody synthesis by the high serum concentration of IgG, thus enhancing suppressor T cell function (474-475), may explain the IgG subclass decrease during normal pregnancy. Serum IgG subclass in women with a history of recurrent miscarriage but whose pregnancies continued showed patterns similar to those in normal pregnant women.

From the results obtained in this study it is possible to hypothesise that the inhibition of immune activity is associated with high levels of serum immunoglobulin. In the first trimester of normal pregnancy, the increased IgG inhibits the immune activity which contributes to the maintenance of a normal pregnancy. In those with recurrent miscarriage whose pregnancy again failed the serum levels of IgG, though increased, were not high enough to interfere with the regulation of the immune responses itself. The interactions between the feedback inhibition and the exact IgG subclass level in serum requires further study. It is also possible that higher levels of IgG are protective in that they reduce an antigenic challenge

which could trigger abortion.

4.4.2 Effect of TPO auto-antibody status on pregnancy outcome

Patients who again aborted were found to have significantly higher titre and avidity of auto-antibody than those who had a successful pregnancy outcome. This suggests that the production of auto-antibodies may be relevant to pregnancy outcome.

4.5 Penetration of bacterial fragments into the host circulation

The preliminary experiments conducted here indicate that bacterial DNA cannot be detected in host serum. However, other studies are required using a wider range of probes in a wider patient group (e.g. pregnant women with severe gingivitis) to verify this conclusion. Moreover, inhibitory effects of host DNA or proteins on the PCR reaction require to be excluded.

4.6 Overall Conclusions

The general aim of this project has been to investigate the relevance of systemic and local humoral immunity to the aetiology and progression of periodontal disease; and to place these findings in the wider context of immune-inflammatory processes.

A correlation has been confirmed between local levels of antibody to *P. gingivalis* and site disease status. Moreover, levels of immunoglobulins and inflammatory mediators have been shown to be different in healthy sites in younger and older individuals, suggesting that these changes may be relevant in the development of periodontal disease as a disorder of ageing. Possible differences between local humoral immune responses at matched oral implant and natural tooth sites have also been highlighted.

The *in situ* hybridisation studies of the gingival plasma cell infiltrate have demonstrated that κ-light chain producing plasma cells predominate in periodontitis lesions and this could be a useful method of distinguishing the stage or activity of periodontitis

lesions, and may differ between different forms of periodontitis.

Levels of IgG1-4 in the GCF have been shown to correlate well with plasma cell proportions in the gingiva, but GCF levels of IgA2 were much higher than IgA2-producing plasma cell counts would indicate. Moreover, high levels of IgA1 F_{ab} fragments in the GCF suggest preferential IgA1 digestion by bacterial proteases.

The antibody avidity studies have demonstrated a correlation between serostatus to *P. gingivalis* and the development of antibody avidity and treatment outcome. This may have a major impact on the management of periodontal therapy.

The studies of obstetric immunology, similarly, have shown correlations between serum levels of IgG1-4 and TPO auto-antibody and pregnancy outcome. This puts the studies in periodontal disease into a wider context, indicating that differences in systemic humoral immunity may be important in the differential diagnosis and management of patients with a range of immune-inflammatory conditions.

This project has illuminated a number of areas which would merit further study. The relevance of antibody avidity and serostatus to *P. gingivalis* requires particular attention in studies with larger patient groups followed prospectively over longer longitudinal monitoring periods.

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List of publications

The following publications contain material directly relevant to this thesis:-

(Copies of certain papers are attached)

1. Kinane DF, Mooney J, MacFarlane TW, McDonald M. Local and systemic antibody response to putative periodontopathogens in patients with chronic periodontitis: Correlation with clinical indices. Oral Microbiol Immunol 1993; **8**: 65-68.

2. Mooney J, Adonogianaki E, Kinane DF. Relative avidity of serum antibodies to putative periodontopathogens in periodontal disease. J Periodont Res 1993; **28**: 444-450.

3. Adonogianaki E, Mooney J, Wennstrom JL, Lekholm U, Kinane DF. Acute-phase proteins and IgG against *P. gingivalis* in peri-implant crevicular fluid: A comparison with gingival crevicular fluid. Clin Oral Impl Res 1995; **6**: 14-23.

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The following publications contain material indirectly relevant to this thesis:-

(Copies of certain papers attached)

1. Haerian A, Adonogianaki E, Mooney J, Docherty AJP, Kinane DF. Gingival crevicular stromelysin, collagenase and tissue inhibitor of metalloproteinases levels in healthy and diseased sites. J Clin Periodontol 1995; **22**: 505-509.

2. Haerian A, Adonogianaki E, Mooney J, Manos A, Kinane DF. Effects of treatment on gingival crevicular collagenase, stromelysin and tissue inhibitor of metalloproteinases and their ability to predict response to treatment. J Clin Periodontol 1996; **23**: 83-91.

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4. Kinane DF, Adonogianaki E, Moughal N, Winstanley FP, Mooney J, Thornhill M. Immunocytochemical

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