CLINICAL AND GENETIC ANALYSIS OF EARLY ONSET PERIODONTITIS

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List of Abbreviations

A	adenine
Aa	*Actinobacillus actinomycetemcomitans*
ACJ	amelocemental junction
AL	attachment loss
AP	adult periodontitis
APC	antigen presenting cell(s)
B7	co-stimulatory molecule for macrophage/T cell antigen presentation
Bf	*Bacteroides forsythus*
BL	alveolar bone loss
bp	base pair(s)
C	constant region of α and β chains of T cell receptor; cytosine
CD	cluster of differentiation antigen(s)
CD2	receptor for lymphocyte function associated antigen 3
CD3	integral part of the T cell receptor complex
CD4	marker for T-helper cell(s)
CD5	marker for B cell clone
CD8	marker for T-suppressor cell(s)
CD45RA	marker for naïve and resting memory T cell
CD45RO	marker for activated memory T cell
cDNA	complementary DNA synthetically manufactured *in vitro*
CI	confidence interval
cM	centimorgans
COX	cyclooxygenase (prostaglandin H synthase)
Cr	*Campylobacter rectus*
D	diversity region of β chain of T cell receptor
DGI	dentinogenesis imperfecta
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	2' deoxyribonucleotide 5'triphosphates
DZ	dizygous
DZA	DZ twins reared-apart
DZT	DZ twins reared-together

Ec	*Eikenella corrodens*
EDTA	ethylene diamine tetraacetic acid
ELAM-1	endothelial leucocyte adhesion molecule-1 (E-selectin)
EOP	early-onset periodontitis
FcγR	Fc gamma receptors on phagocytes
FMLP	N-formyl-methyl-leucyl-phenylalanine
Fn	*Fusobacterium nucleatum*
G	guanine
GCF	gingival crevicular fluid
GEOP	generalised early-onset periodontitis
GMC	gingival mononuclear cells
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPP	generalised prepubertal periodontitis
HA	hyaluronic acid
H/G	healthy/gingivitis
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HSP	heat shock proteins
ICAM-1	intercellular adhesion molecule-1
IEOP	incidental early-onset periodontitis
IFNγ	interferon gamma
IL	interleukin
IL1A	IL-1α gene
IL1B	IL-1β gene
IL-1R	IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IL1RN	IL-1ra gene
J	joining domain of α and β chains of T cell receptor
JE	junctional epithelium
kDa	kilodalton
LAD	leucocyte adhesion deficiency syndrome
LECAM-1	lectin adhesion molecule-1 (L-selectin)
LEOP	localised early-onset periodontitis
LFA	lymphocyte function-associated antigen

LPS	lipopolysaccharide
LTA	lymphotoxin-α gene
LTB	lymphotoxin-β gene
LPP	localised prepubertal periodontitis
MBG	molecular biology grade
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MMP	matrix metalloproteinase(s)
mRNA	messenger ribonucleic acid
MSF	migration stimulation factor
MZ	monozygous
MZA	MZ twins reared-apart
MZT	MZ twins reared-together
NK	natural killer cell(s)
NUG	necrotising ulcerative gingivitis
OR	odds ratio
PBMC	peripheral blood mononuclear cell(s)
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule-1
Pg	*Porphyromonas gingivalis*
PGE_2	prostaglandin E_2
Pi	*Prevotella intermedia*
PIC	polymorphism information content
PLS	Papillon-Lefèvre syndrome
PMN	polymorphonuclear leucocyte(s)
PP	prepubertal periodontitis
RA	rheumatoid arthritis
RFLP	restriction fragment length polymorphism
RPP	rapidly progressing periodontitis
SDS	sodium dodecyl sulphate
sIL-1R	soluble IL-1 receptor
SLE	systemic lupus erythematosus
ST	smokeless tobacco

sTNF-R	soluble TNF receptor
T	thymine
TAP	transporter associated with antigen processing
TCR	T cell receptor
TDT	Transmission disequilibrium test
TEMED	tetramethylethylenediamine
Th	T-helper cell subset
TNF	tumour necrosis factor
TNFA	TNFα gene
TNFB	TNFβ gene (LTA)
TNF-R	TNF receptor
UV	ultra-violet
V	variable region of α and β chains of T cell receptor
VNTR	variable number of tandem repeats

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Declaration

This thesis is the original work of the author.

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Summary

The term early-onset periodontitis (EOP) represents a collection of diseases diagnosed in children, adolescents and young adults. These disorders are aetiologically and clinically heterogeneous. They have a low frequency in the general population but when they do occur can result in rapid destruction of periodontal support. Without treatment tooth loss often ensues.

The major aetiological agent of periodontitis is microbial plaque. However, other factors also predispose to disease. These include smoking, systemic diseases and stress. Most of the tissue destruction seen in periodontitis is thought to be the result of an inappropriate host response to the bacterial challenge. Certain aspects of the inflammatory and immune response, such as cytokine levels and antibody titres, have been shown to vary between individuals. Convincing evidence now exists for a genetic susceptibility to periodontal disease. This is particularly strong for EOP.

One method of investigating the genetic basis of a disease is through studies of large cohorts of unrelated individuals. The frequencies of polymorphisms of candidate genes whose protein products play a role in the disease process can be compared between cases and controls using recently developed molecular genetic techniques. A significant difference in the frequency of a specific polymorphism between a disease group and a control group is evidence that the candidate gene plays some role in determining susceptibility to disease. An association indicates that either the candidate gene directly affects disease susceptibility or is in linkage disequilibrium with the disease locus.

The research reported in this thesis has applied this method to the study of genetic polymorphisms in the *HLA* (Human Leucocyte Antigen) complex and the *IL10* gene in generalised early-onset periodontitis (GEOP). The polymerase chain reaction (PCR) technique was employed in order to amplify an atypical restriction site in the *HLADQB1* gene using sequence specific oligonucleotide primers. The resulting products were subjected to restriction fragment length polymorphism (RFLP) analysis. The fragments obtained were the undigested 1000 base pair (bp) product representative

of allele two and the digested 700 and 300 bp fragments representative of allele one. The fragments were separated using agarose gel electrophoresis. Subjects were either homozygous for allele two or heterozygous. No differences in frequency of the presence or absence of the atypical restriction site were found between patients or controls.

PCR was also used to investigate microsatellites in the tumour necrosis factor (TNF) gene and the *IL10* promoter region. Variable number of tandem repeats were amplified and used to distinguish the different alleles. The resulting products were separated according to size using denaturing polyacrylamide gel electrophoresis. The results of these studies showed no significant associations between any of the candidate genes investigated and GEOP. However, trends were observed in the occurrence of alleles at the *IL10G* and *TNFa* microsatellites. A decrease in *IL10G9* and an increase in *IL10G13* in patients compared with controls were noticed. When smoking was included as a co-variate for the patient group the trend seen at this locus was found to be due to the smokers. An increase in *TNFa2* was also seen in GEOP, although there were no differences in frequency between smokers and non-smokers. In addition, a significant association was found between the *TNFa2TNFd4* allelic combination and GEOP, which had previously been identified as a disease genotype in Crohn's disease. These observations warrant further investigation. However, they also highlight the problems of investigating candidate genes in EOP. Since EOP is a genetically and environmentally heterogeneous group of diseases, finding convincing associations with specific polymorphisms in populations of unrelated individuals is extremely difficult, even if the case and control groups have been racially matched.

Segregation analyses of families with a high prevalence of EOP have proposed X-linked dominant, autosomal dominant and autosomal recessive modes of transmission. One of the major problems of genetic studies of EOP is the clinical classification of relatives. Since signs and symptoms of EOP may not be seen until adolescence or later, and because EOP can be confused with adult periodontitis (AP) after the age of 35 years, definitive diagnosis is difficult. In addition, multiple tooth loss in patients within the age range may result from other reasons than periodontitis. Some

individuals are irregular attenders and general dental practitioners often fail to keep accurate records.

Because of the difficulty of diagnosis of relatives of EOP probands outlined above, confusion exists between different research groups with regard to the clinical classification of EOP. This may affect the results of genetic model testing in families with an increased frequency of EOP. One of the studies presented in this thesis has attempted to define and validate an index which is suitable for the current and retrospective diagnosis of family members. The index was applied to a large family with fourteen affected individuals. Segregation analysis of this kindred supported the autosomal dominant theory of inheritance of GEOP. The ultimate aim of the clinical and genetic analysis of the large kindred presented here is to carry out a family linkage study. Therefore, in addition to the segregation analysis, an analysis of the power of the pedigree to detect linkage was performed. The phenocopy rate was also increased in order to take account of possible environmental aetiological factors. It was found that even with a phenocopy rate of 15% the pedigree would still be informative for linkage analysis.

The future identification of genetic markers of susceptibility to periodontal disease could have wide-ranging health and economic implications. Targeting preventive measures at individuals most likely to benefit from them, instead of employing a broad-based approach to the treatment of periodontitis would preserve valuable financial and healthcare resources. During the last five years a number of researchers have suggested that periodontal disease may predispose to atherosclerosis and coronary heart disease. If these initial findings are corroborated, the identification of risk markers for periodontal disease will become increasingly important in the prevention and treatment of cardiovascular disorders.

Chapter 1 Introduction

1.1 General Introduction

Over the past thirty to forty years man's understanding of the aetiology and pathogenesis of periodontal disease has advanced enormously. During the 1960s and 1970s periodontitis was believed to be a purely infectious disease (Page and Schroeder, 1982). Both specific and non-specific microbial aetiologies were proposed (Loesche, 1979; Theilade, 1986). Chronic periodontitis was estimated to be universally prevalent and to affect almost all of the adult population by the age of thirty-five to forty years (Papapanou and Lindhe, 1997). More than 90% of the variation in disease severity was considered to be due to age and oral hygiene status.

Since the 1980s, it has become clear that the effectiveness of the host's inflammatory and immune response to plaque bacteria also plays an important role in susceptibility to disease (Kinane and Lindhe, 1997). Some researchers believed that periodontal destruction was caused entirely by a defective immune system, since individuals with healthy periodontiums also harboured suspected periodontal pathogens. At the same time epidemiological surveys from around the world, contrary to earlier reports, began to show that a minority of the population (albeit a substantial one) were affected by severe disease (Hugoson and Jordan, 1982; Löe *et al.*, 1986; Baelum, Fejerskov and Manji, 1988; Jenkins and Kinane, 1989; Burt, 1991; Brown and Löe, 1993). This level of disease could lead to tooth loss and compromise aesthetics, comfort and function. However even within susceptible individuals the extent of periodontal destruction was not uniform throughout the dentition and was also site-specific (Baelum, Fejerskov and Manji, 1988). The majority of individuals appeared not to be susceptible to severe disease. In addition some subjects were resistant to periodontal disease despite the presence of large plaque deposits over many years (Löe *et al.*, 1986; Baelum, Fejerskov and Manji, 1988).

Included within the susceptible group were individuals with the early-onset forms of periodontitis, which are the subject of this thesis. Towards the end of the 1980s the

idea of a possible underlying genetic predisposition for EOP re-emerged. Family histories of EOP had been recorded as early as the 1920s, but the concept of a hereditary component to the disease fell into disrepute during the 1950s (Hassell and Harris, 1995).

In the 1990s more evidence to support a genetic basis for periodontal disease has been reported in the literature (Michalowicz *et al.*, 1991a; Michalowicz, 1994; Marazita *et al.*, 1994; Hart and Kornman, 1997). However, the importance of other factors in the aetiology of the disease such as smoking and the presence of specific bacteria has also been highlighted (Grossi *et al.*, 1994; Barbour *et al.*, 1997). In addition the contribution of the subgingival microbiota to the aetiology of periodontitis has been investigated more fully (Socransky and Haffajee, 1997). Products produced by highly toxic strains of periodontal bacteria may cause tissue damage directly.

As we move towards the next millennium, the consensus is that the periodontal diseases are a complex heterogeneous group of related diseases with a multifactorial aetiology. Bacteria are essential to cause disease but may only do so in a susceptible host. In addition many other factors including lifestyle variables such as smoking, oral hygiene, diet and stress and also systemic disease and race may affect the clinical manifestation of periodontitis (Kinane and Davies, 1990; Genco, 1996; Offenbacher, 1996; Moss *et al.*, 1996; Barbour *et al.*, 1997; Schenkein, 1998). It has been suggested that these disease modifiers can interact with different aspects of the host response and the microbial challenge. The nature of the immune response may vary between patients but manifest the same clinical symptoms. It is possible that the quality of an individual's antibody response may influence the outcome of treatment (Mooney *et al.*, 1995). Various characteristics of the host response, which play a part in tissue homeostasis in the periodontium, are under genetic control.

This chapter reviews the current literature of relevance to the studies presented in this thesis. Although the subsequent chapters are concerned with genetic analysis of EOP, background knowledge of the aetiology and pathogenesis of periodontitis are included to place this work in context.

1.2 The Aetiology and Pathogenesis of Periodontitis

1.2.1 Introduction

The following review is not intended to be a comprehensive analysis of the literature. It is instead a general overview with an emphasis on particular areas relevant to the research reported in this thesis. Topics of special interest, discussed in separate sections, include the role of cytokines in the inflammatory and immune response and the major histocompatibility complex.

In order to understand the processes involved in the aetiology and pathogenesis of periodontitis, it is important to have some knowledge of the anatomy of the periodontium. The periodontium consists of the gingivae, the cementum, the periodontal ligament and the alveolar bone. The gingivae are made up of the keratinised oral epithelium, the oral sulcular epithelium and the thin junctional epithelium (JE) which is about fifteen cell layers thick. The gingivae have a core of connective tissue with a vascular network subjacent to the epithelium. The JE is highly permeable to the passage of cells and proteins of the immune system into the gingival crevice. The exudate produced in the gingival crevice, constitutes serum and plasma proteins that have passed from the gingival capillaries through the tissues and, is known as gingival crevicular fluid (GCF) (Egelberg, 1967; Cimasoni, 1983). Normal healthy gingiva should be pale pink in colour and have a firm texture. In some individuals it has a stippled appearance. The margin should have a scalloped outline and be well adapted to the tooth surface. The gingivae should not bleed on gentle probing into the gingival sulcus. The coronal margin should terminate in a knife-edge between the tooth and soft tissue.

Histologically normal clinically healthy gingiva shows evidence of a mild inflammatory infiltrate consisting of small numbers of leucocytes, predominantly polymorphonuclear leucocytes (PMN), migrating towards the gingival sulcus (Page and Schroeder, 1976). In addition monocytes, macrophages and lymphocytes, can be seen in the underlying connective tissue. This situation exists where the gingival margins have been kept relatively plaque free and is the realistic scenario. An alternative super healthy situation has been described where the gingival sulcus may be

absent. The JE is attached to the tooth surface by hemidesmosomes and supported by dense collagen bundles in the underlying connective tissue. This can be accomplished by chemical plaque control or by professional supragingival hygiene therapy. Kinane and Lindhe (1997) defined this situation as 'pristine gingiva'. Even in the pristine state, very small numbers of leucocytes (neutrophils and macrophages) can be seen in the JE. A few isolated lymphocytes and plasma cells may be associated with the sub-epithelial vascular network and in the deeper connective tissues (Attström, Graf-de-Beer and Schroeder, 1975; Payne *et al.*, 1975). These cells do not constitute an infiltrate; microscopically and clinically no manifestations of tissue damage are evident (Page and Schroeder, 1976). In contrast normal healthy gingiva does show signs of up to 5% loss of connective tissue volume (Kinane and Lindhe, 1997).

1.2.2 Microbial Aetiology

Evidence for a microbial aetiology to gingivitis comes from reports of healthy gingival microflora and from naturally occurring and experimental gingivitis (Löe, Theilade and Jensen, 1965; Slots, 1977; Savitt and Socransky, 1984; van der Weijden *et al.*, 1994). Studies of experimental gingivitis demonstrated changes in the composition and structure of plaque preceding the developing gingival inflammation. A shift in the microflora from one dominated by Gram positive cocci and rods to one showing marked evidence of Gram negative anaerobic rods and spirochaetes was documented (Löe, Theilade and Jensen, 1965; Theilade *et al.*, 1966). In the active phase of periodontitis the proportion of Gram negative rods increases to about two thirds of the subgingival plaque. *Porphyromonas gingivalis* (Pg), *Bacteroides forsythus* (Bf) and *Actinobacillus actinomycetemcomitans* (Aa) have been strongly associated with the various forms of destructive periodontitis (Socransky and Haffajee, 1997). Particular clonal types of these bacteria have the ability to initiate destruction of the connective tissue matrix. For example Pg produces potent enzymes such as collagenase. However most of the proteases present in periodontal lesions are of host origin. The bacterial toxins stimulate the host cells, which bring about the observed destruction (Page *et al.*, 1997).

It was assumed in the past that gingivitis progressed with time to periodontitis (Russell, 1956). Evidence from animal studies indicated that periodontitis was always preceded by gingivitis (Saxe *et al.*, 1967; Lindhe, Hamp and Löe, 1973) and did not occur when microbial plaque was removed on a daily basis (Lindhe, Hamp and Löe, 1975). However gingivitis may persist without ever progressing to periodontitis (Lindhe, Hamp and Löe, 1975; Page and Schroeder, 1982). Furthermore recent evidence indicates that individuals susceptible to periodontitis may have a very short or indistinguishable gingivitis stage, before progressing to periodontitis (Page *et al.*, 1997). It appears that gingivitis and periodontitis may in fact be different clinical presentations of the same pathological process.

1.2.3 Pathogenesis

1.2.3.1 Introduction

The clinical signs of gingivitis are usually manifested within ten to twenty days of plaque accumulation (Kinane and Lindhe, 1997). The gingival tissues become red and swollen. The gingival sulcus may have an increased tendency to bleed on gentle probing. The normal gingival contour changes and the surface of the gingivae may become smooth with the loss of stippling. As a result of the swelling, a gingival pocket forms where subgingival plaque may accumulate and aggravate the situation still further. Hormonal changes such as those that occur at puberty, during pregnancy and when taking the contraceptive pill, can exacerbate inflammation and swelling and cause marked gingival hyperplasia. Certain drugs (phenytoin, cyclosporin and the calcium channel blockers) may also produce similar effects (Seymour, Thomason and Ellis, 1996).

Page and Shroeder (1976) divided the histopathological stages of the development of gingivitis and periodontitis into the initial, early, established and advanced lesions. This designation will be used as a framework for the following discussion. It is important to be aware however, that based on recent evidence, the early lesion, with an infiltrate dominated by lymphocytes, does not exist in adults as a distinct entity. It

does occur in children around erupting teeth and in chronic gingivitis in adolescents (Page *et al.*, 1997).

1.2.3.2 The initial lesion

The initial lesion emerges within two to four days of plaque accumulation. Vasodilation and hyperpermeability of the gingival capillaries occurs, with a concomitant acute exudative inflammatory response. It has been shown that endothelial cells, resident connective tissue cells and JE cells, upregulate the expression of adhesion molecules under the influence of proinflammatory cytokines (Kinane *et al.*, 1991; Moughal *et al.*, 1992; Takahashi *et al.*, 1994; Gemmell *et al.*, 1994; Gemmell, Sved and Seymour, 1995). Intercellular adhesion molecule-1 (ICAM-1) and endothelial leucocyte adhesion molecule-1 (ELAM-1) encourage the aggregation of leucocytes in the tissues and the passage of PMN through the tissues and into the gingival sulcus (Kinane *et al.*, 1991; Moughal *et al.*, 1992). Watanabe and co-workers (1991) demonstrated increased expression of ICAM-1 receptor on crevicular fluid PMN from healthy gingiva compared with peripheral blood PMN, giving further credence to the role of adhesion molecules in the inflammatory response.

The initial lesion is localised in the junctional epithelium and the most coronal region of the gingival connective tissue. It occupies no more than 5 to 10% of the connective tissue. It is possible that the initial lesion is indistinguishable from the normal situation or in fact from the early lesion. Clinically healthy gingivae may manifest a pre-established chronic lymphoid infiltrate, as seen in the early lesion, which on the initiation of gingival inflammation responds with an exacerbation of an acute exudate superimposed on the lymphoid infiltrate (Page and Schroeder, 1976).

1.2.3.3 The early lesion

The early lesion appears within four to seven days of plaque accumulation (Payne *et al.*, 1975). The number of vessels in the sub-epithelial plexus increases due to the opening up of previously inactive capillary beds (Egelberg, 1967). The early lesion is characterised by a dense lymphoid infiltrate that makes up 75% percent of the total

infiltrate population. There are numerous immunoblasts within the area and plasma cells if present are few in number and found at the periphery of the lesion (Listgarten and Ellegaard, 1973; Payne *et al.*, 1975; Seymour *et al.*, 1983; Seymour, Powell and Aitken, 1983; Brecx *et al.*, 1987). The infiltrated area may occupy up to 15% of the connective tissue. Fibroblasts within the lesion degenerate, probably by apoptosis, and up to 60 to 70% collagen destruction occurs in order to make space for the infiltrating leucocytes (Page and Schroeder, 1976; Kinane and Lindhe, 1997). Inflammatory changes are detected clinically by this stage (Löe, Theilade and Jensen, 1965). The acute, exudative, inflammatory response persists in the early lesion, and reaches a peak, and levels off between six and twelve days after the onset of clinical gingivitis (Lindhe, Hamp and Löe, 1973). The junctional and sulcular epithelium proliferates and produces rete ridges, which project into the underlying connective tissue (Schroeder, Münzel-Pedrazzoli and Page, 1973).

1.2.3.4 The established lesion

Page and Schroeder (1976) defined the established lesion as one dominated by plasma cells within the infiltrated connective tissue, without evidence of extensive alveolar bone loss (BL). Plasma cells are mainly situated in the coronal connective tissue but are also found clustered around blood vessels. A few cells are found in the deeper connective tissue. Other features of the established lesion include the persistence of the inflammatory exudate; the presence of immunoglobulins in the connective tissue and JE; the continued loss of collagen in the area of infiltration and fibrosis and scarring at sites removed from the region of destruction; and the progressive proliferation, apical migration and lateral extension of the JE, in an attempt to preserve epithelial integrity (Page and Schroeder, 1976). The coronal part of the JE may evolve into pocket epithelium and as a result become separated from the tooth surface, thus deepening the gingival crevice. If pocket epithelium is present vascular loops can be found, separated from the surface by only one or two epithelial cells, which may in some areas be ulcerated. The pocket epithelium allows easy access for microbial products to the underlying connective tissue and hastens the progression towards periodontitis.

Early investigators believed that the established lesion with a predominant plasma cell infiltrate was evident within two to three weeks of plaque accumulation (Page and Schroeder, 1976). More recently Page and Shroeder (1982) suggested that in severe gingivitis lymphocytes still predominated over plasma cells and the T:B cell ratio was almost equal to one. Subsequently an experimental gingivitis study found that there was no increase in B cell numbers over the 21 days of the study (Seymour *et al.*, 1988). In another longitudinal study in adults, Brecx *et al.* (1988) demonstrated that even after six months of neglected oral hygiene the predominant cell types were granulocytes and lymphocytes. The number of plasma cells increased over the time period to 10% of total cell volume. This could reflect individual susceptibility since some adults seem to be resistant to established gingivitis (van der Weijden *et al.*, 1994).

1.2.3.5 The advanced lesion

Clinical signs of the advanced lesion include periodontal pocket formation, suppuration, fibrosis of the gingivae, attachment loss (AL), tooth mobility and migration, and eventual tooth exfoliation (Page and Schroeder, 1976).

The advanced lesion manifests all the features of the established lesion but also includes destruction of the connective tissue attachment to the tooth, apical migration of the JE, and BL (Schroeder and Lindhe, 1975; Page and Schroeder, 1976; Lindhe, Liljenberg and Listgarten, 1980; Okada, Kida and Yamagami, 1983; Zappa *et al.*, 1992). The lesion is no longer localised and may extend apically and laterally to form a wide band of infiltrated connective tissue, around the necks and roots of the teeth. Despite the destruction of the collagen fibre bundles of the marginal gingiva, the transseptal fibre bundles appear to be constantly regenerated as the lesion progresses apically. This has the effect of separating the progressing alveolar bone destruction from the lesion in the coronal gingiva. Whereas the infiltrated connective tissue is virtually free of collagen fibres, dense fibrotic tissue is present at more distant sites and in association with areas of bone resorption.

1.2.4 Susceptibility to Periodontitis

There are many similarities histopathologically between the established and advanced lesions. A number of investigators have therefore looked for subtle differences in cell number or type, which might indicate why progression to destructive periodontitis occurs. The general consensus is that a change from a T cell dominated to a B cell and plasma cell dominated lesion, precedes the development of destructive periodontitis (Mackler *et al.*, 1977; Seymour, Powell and Davies, 1979; Seymour and Greenspan, 1979; Page and Schroeder, 1982; Reinhardt *et al.*, 1988a).

Zappa and co-workers found that there was a significantly increased number and percentage of overall inflammatory cells, macrophages, plasma cells, mast cells and lymphocytes, in periodontal sites which had recently undergone rapid destruction. Active sites were compared with matched non-progressing control sites. A significant decrease in the number and percentage of fibroblasts was also observed (Zappa *et al.*, 1990; 1991). More recently other groups have confirmed the above findings (Gemmell *et al.*, 1994; Liljenberg *et al.*, 1994; Yamazaki, Nakajima and Hara, 1995). Liljenberg *et al.* (1994) found the density of plasma cells was increased from 31% in inactive sites to 51.3% in active sites. In addition the proportion of macrophages increased from 0.3% to 2.1%. Gemmell *et al.* (1994) recorded a decrease in the mean ($\pm$ standard error) of the T:B cell ratio in gingival biopsies taken from healthy/gingivitis and moderate/severe periodontitis patients with increasing size of infiltrate. The ratio decreased from 4.63 ($\pm$ 1.25), in areas adjacent to the sulcular and junctional epithelium (small infiltrates), to 0.76 ($\pm$ 0.16) in infiltrates extending throughout the whole of the gingiva. The latter situation was only found in patients with moderate or severe periodontitis. It seems possible that the established lesion with extensive plasma cell infiltration may in fact be early periodontitis (Kinane and Lindhe, 1997). Support for this theory, as previously mentioned, comes from the study of long-term gingivitis in man. In this report a lesion dominated by plasma cells did not occur even after six months of plaque accumulation (Brecx *et al.*, 1988).

It has been suggested that in resistant patients neutrophils and antibodies are able to contain the destructive lesion (Page *et al.*, 1997). Susceptibility to periodontitis may

depend on the ability of Gram negative bacteria to circumvent neutrophil clearance. It has been shown that Pg lipopolysaccharide (LPS) does not activate the expression of adhesion molecules and chemoattractants by endothelial cells, fibroblasts and epithelial cells. It also blocks E-selectin expression by other bacteria and their LPS. This would allow the passage of LPS into the tissues, which would trigger the release of cytokines and proteases from resident tissue cells, macrophages and T cells. The T cell response controls the inflammatory response and humoral immunity. Antibodies enhance the effectiveness of neutrophils, which reduces the microbial challenge and the levels of LPS (Page *et al.*, 1997). A dense plasma cell infiltrate could signify an inability to raise a successful immune response against periodontal pathogens (Kinane and Lindhe, 1997). The antibodies produced may be either ineffective at eliminating the bacteria or result in the formation of immune complexes which result in local tissue damage, including triggering fibroblast apoptosis. In a recent study, a higher proportion of T cells was demonstrated in EOP and gingivitis sections than in AP lesions, but the reverse was the case for B cells (Lappin *et al.*, 1999). The authors suggested that EOP lesions might be at an earlier stage in the disease process; alternatively different immunopathological backgrounds may exist for AP and EOP.

It has been proposed that decreased numbers of fibroblasts may be the best way of differentiating between progressing and non-progressing sites (Zappa *et al.*, 1992). Schor and co-workers (1996) have also recently shown that healthy gingival fibroblasts resemble foetal fibroblasts, with regard to cell migration and hyaluronic acid (HA) synthesis. These two effects are modulated by migration stimulation factor (MSF), which is produced by foetal fibroblasts but not by the adult phenotype. It appears that MSF stimulates HA synthesis, and the accumulation of HA in the collagen matrix results in activation of cell migration. It has been suggested that alteration in the phenotype of fibroblast subpopulations in the gingivae to an "adult-like cell", may have an influence on progression of periodontal disease (Schor *et al.*, 1996; Page *et al.*, 1997). One possible mechanism might be through influencing apical downgrowth of JE (Mackenzie *et al.*, 1991). It has also been demonstrated that under disease conditions fibroblasts switch from producing collagens and tissue inhibitors of matrix metalloproteinases (MMP) to synthesising and releasing MMP (Zappa *et al.*, 1992). They may also produce their own inflammatory mediators such as interleukin

(IL)-1β (Page *et al.*, 1997). Takiguchi *et al.* (1997) have shown that increased production of IL-1β by "old" fibroblasts, compared with "young cells", occurred in the presence of LPS from *Campylobacter rectus* (Cr). The higher levels of IL-1β also correlated with increased prostaglandin E_2 (PGE_2) release from "old" fibroblasts. Furthermore Wassenaar *et al.* (1997) have shown gingival fibroblasts able to influence infiltrating T cells in periodontal lesions of AP patients. Interferon gamma (IFNγ)-treated fibroblasts act as antigen-presenting cells for superantigen mediated T cell proliferation. However fibroblasts were not able to present whole-cell antigens of periodontal pathogens and inhibited presentation of these antigens by professional APC. Addition of IL-2 overcame this inhibitory effect.

1.2.5 Host Responses

1.2.5.1 Defensive

Several periodontal pathogens have been associated with potent virulence factors, which may even cause death when injected parenterally into experimental animals (Neiders *et al.*, 1989). In addition, it has been suggested that the selective gain of certain virulence factors may be increased or diminished by local interaction with other bacteria in the periodontal pocket (Socransky and Haffajee, 1992). As a result of these findings, the importance of the host response in localising the infection to the periodontal tissues has been highlighted (Genco, 1992). Although toxins and proteases produced by plaque bacteria can pass from the crevice into the gingival connective tissue, the following defence mechanisms serve to protect the intact gingival sulcus (Page, 1990):

1. The integrity of the epithelium and epithelial attachment, in which keratinocytes may play a major role (Nunes *et al.*, 1994).

2. The flushing action of GCF and the antimicrobial effects of opsonins, antibodies, complement and other plasma components found within it.

3. Local antibody production.

4. The constant turnover of sulcular epithelial cells.

5. The ever present low level inflammatory exudate containing PMN and other leucocytes.

The early events, which lead to an inflammatory and/or immune response, are not fully understood (Tonetti, 1993). Mounting an effective response depends upon transmission of inflammatory stimuli to the endothelial cells of the sub-epithelial gingival plexus. This is thought to occur by the passage of bacterial antigens into the intact epithelium. Alternatively stimulation of intraepithelial cells, such as keratinocytes, Langerhans or dendritic cells, and specific subsets of T cells, by direct contact with bacteria or their products is another possible hypothesis (Nunes *et al.*, 1994). Keratinocytes may respond to bacterial stimuli by producing cytokines, adhesion molecules and chemotactic factors. However HLA-DR+ keratinocytes have also been found to elicit a state of anergy and tolerance of T cells when exposed to bacterial antigens (Walsh, Seymour and Powell, 1986). The function of the other cell types is at present uncertain, but they increase at sites of inflammation and may be involved in limiting penetration of antigens and in antigen presentation (Page *et al.*, 1997).

Endothelial cells in turn, once activated, also upregulate the expression of adhesion molecules as has already been mentioned earlier (Kinane *et al.*, 1991; Moughal *et al.*, 1992; Gemmell *et al.*, 1994). PMN, monocytes and lymphocytes, constantly migrate from the capillaries towards the gingival sulcus. They are under the influence of chemoattractants of host origin, such as IL-8, monocyte chemoattractant protein 1 (MCP-1), complement 5a and leukotriene B4, and microbial toxins (Bickel, 1993; Tonetti, 1993; Tonetti *et al.*, 1994). Adhesion molecules found in the junctional epithelium also aid the passage of PMN towards the gingival crevice. Tonetti and co-workers (1998) recently provided evidence of a transepithelial gradient of IL-8 and ICAM-1 guiding PMN towards the junctional epithelial surface and the area nearest to bacterial antigens. Initially passage of PMN occurs through the junctional epithelium and the pocket epithelium. As the size of the infiltrate increases the junctional, sulcular and oral epithelium may all express ICAM-1 (Gemmell *et al.*, 1994). Pocket epithelium can also be activated to produce PGE_2 and MMP, which aid the initiation

and progression of the inflammatory response in the adjacent connective tissue (Page *et al.*, 1997).

Lymphocytes are thought to be retained in the tissues by cell-cell and cell-matrix adhesion molecules (Hayashi *et al.*, 1994; Takahashi *et al.*, 1994; Gemmell *et al.*, 1994; Gemmell, Sved and Seymour, 1995). An increase in the percentage of ICAM-1, platelet endothelial cell adhesion molecule-1 (PECAM-1) and lectin adhesion molecule-1 (LECAM-1) positive infiltrating cells, with increasing size of inflammatory infiltrate, has been demonstrated (Gemmell *et al.*, 1994). However with worsening inflammation expression of ICAM-1 by junctional epithelium keratinocytes adjacent to the infiltrate was not evident. Loss of the chemoattractant gradient may lead to retention of phagocytes within the tissues, where they are activated and release MMP. In addition, loss of the chemoattractant gradient leads to direct exposure of the tissues to LPS and other bacterial toxins (Page *et al.*, 1997).

Another interesting finding in the study of Gemmell *et al.* (1994) was the low number of endothelial cells expressing ELAM-1, in contrast to results of studies of chronic dermatoses. It has been found that ELAM-1 mediates adhesion of a sub-population of memory T cells found in high numbers in chronic dermatoses and low numbers in extra-cutaneous areas of chronic inflammation (Picker *et al.*, 1991). It is possible that ELAM-1 is upregulated in early gingival lesions to encourage the migration of PMN into the area. In periodontitis lesions dominated by lymphocytes, ELAM-1 expression disappears. This suggests that T cell clones vary between different sites of chronic inflammation. It has been found that heterogeneity and selective localisation of T cell clones exists, between human skin, gingival mucosa and peripheral blood, in healthy subjects (Kinane *et al.*, 1993).

1.2.5.2 T cells

T cells are present at all stages of the development of the chronic periodontal lesion (Page and Schroeder, 1982; Okada, Kida and Yamagami, 1983; Reinhardt *et al.*, 1988b). T cell responses to oral pathogens may depend upon presentation of antigens by macrophages and Langerhans cells, in the context of HLA class II antigens (Nunes *et al.*, 1994). It appears likely that antigen presenting cells (APC) transport antigens to

the local lymph nodes, where presentation to naïve T cells takes place, and proliferation of primed memory cells follows. The memory cells then circulate from the lymph nodes to the gingivae under the influence of bacterial chemoattractant products in the tissues and specific endothelial determinants (Wynne, Walsh and Seymour, 1988; Taubman *et al.*, 1994). This process has been described as homing. Support for this theory comes from studies of T cell subsets expressing specific Vβ receptors (Mathur *et al.*, 1995; Yamazaki *et al.*, 1996; 1998; Nakajima, Yamazaki and Hara, 1996; Gemmell *et al.*, 1997; Berglundh *et al.*, 1998). In addition a lack of proliferating cells in periodontitis lesions has been demonstrated (Takahashi, Lappin and Kinane, 1996; Koulouri *et al.*, 1999).

Nearly all B cell responses are considered to be T cell dependent. A significant positive correlation between IL-4 producing cells and the B:T cell ratio in periodontitis sections has been demonstrated (Yamazaki, Nakajima and Hara, 1995). However, proliferation to B cell mitogens has not always correlated with absolute T cell counts or the relative proportions of specific subsets (Engel *et al.*, 1984). It seems likely that regulation of B cell responses may be influenced more by the cytokine profile of T cells than by the numbers or proportions of T cell subsets (Mathur and Michalowicz, 1997).

Different cell types may be differentiated histologically by cell-surface molecules, which are identified by a specific group of monoclonal antibodies. These molecules are known as cluster of differentiation antigens and designated CD. Changes in the relative number of T-helper (CD4):T-suppressor (CD8) cells are considered to be a sign of altered immune regulation. Studies of CD4:CD8 ratios in the peripheral blood and gingival tissues of EOP or AP patients have yielded conflicting results, probably due to wide individual variability (Takahashi *et al.*, 1995; Mathur and Michalowicz, 1997). In addition it has been found that human T cell lines reacting to different periodontal pathogens give rise to different proportions of CD4 and CD8 cells, depending on the bacteria used to establish the cell line (Ishii, Mahanonda and Seymour, 1992). Therefore, the equivocal findings in studies of the proportions of CD4 and CD8 cells in periodontal tissues may not only be due to different levels of disease activity and variation between subjects. Antigens that activate specific T cell

subsets may be bacterial-species specific (Yamazaki *et al.*, 1998). An immunosuppressive factor secreted by Aa has been identified which induces a specific population of activated dual-positive CD4+CD8+ cells (Shenker, Vitale and King, 1995). These cells do not express natural killer cell (NK) markers and may be responsible for inhibiting T- and B cell activation *in vitro*.

Although it appears that the number of CD4 and CD8 cells increases in periodontal lesions when compared with peripheral blood and healthy sites, the ratio of CD4:CD8 cells seems to be depressed (Cole, Seymour and Powell, 1987; Stoufi *et al.*, 1987; Seymour *et al.*, 1997). This is partly due to a relative reduction in CD4 cells compared with CD8 cells, but also because of a small rise in the number of CD8 cells (Stoufi *et al.*, 1987). CD8 cells have been found to down-regulate antibody production in an antigen specific manner (Taubman *et al.*, 1991). However, more recently Wassenaar *et al.* (1996) suggested that CD8 cells might contribute to the local response by suppressing cell-mediated immunity and stimulating humoral immunity. It has been found that the CD4:CD8 ratio varies within the lesion and with the volume of the inflammatory infiltrate (Gemmell and Seymour, 1995). One study also found that reduced local CD4:CD8 ratios correlated well with deeper probing pocket depths in the same disease category (Stoufi *et al.*, 1987). Taken together the results of the above studies indicate that any defect in immune response is restricted locally to the affected periodontal tissues and may not be revealed in the peripheral blood.

Naïve and memory T cells may be recognised by the markers they express. CD45RA identifies naïve (or resting memory) T cells and CD45RO activated memory T cells. Activated memory T cells respond to antigen by triggering intense CD8 cell proliferation, which suppresses antigen specific B cell responses. Naïve T cells tend to respond by suppressing polyclonal antibody production (Taubman *et al.*, 1991). The number of activated memory T-helper cells (CD4+ CD45RO+) in the peripheral blood of patients with AP are significantly elevated above the levels found in periodontally healthy individuals. In diseased tissues most of the CD4 cells are CD45RO+ (Gemmell, Feldner and Seymour, 1992; Yamazaki *et al.*, 1993; Kinane *et al.*, 1998; Lappin *et al.*, 1999). However it has also been shown that a proportion of these cells are also CD45RA+, which suggests reactivation of the memory cell population by

specific, but as yet unknown antigens, in the tissues (Gemmell and Seymour, 1994; Seymour *et al.*, 1997). It appears therefore, that activated cells in these tissues are memory cells, which have been re-stimulated, rather than activated, naïve cells (Taubman *et al.*, 1994). Once cells are activated they acquire certain specific surface molecules, which act as ligands for adhesion molecules expressed by resident tissue cells. Memory cells respond to a particular antigen, and therefore retention of antigen-specific memory cells in the tissues ensures a rapid response to a secondary infection by the same antigen (Mathur and Michalowicz, 1997).

When memory T- and B cells are re-stimulated with antigen they express a number of surface markers. On T cells these include HLA-DR (a class II HLA molecule), CD25 (IL-2 receptor), CD29 (possibly involved in cell-cell adhesion of antigen specific T- and B cells) and CD38 (nicotinamide adenine dinucleotide glycohydrolase – augments B cell proliferation). Lipopolysaccharide and other antigens of periodontal pathogens induce IL-2 receptor expression on peripheral blood mononuclear cells (PBMC) *in vitro*. Monocytes regulate this expression, since IL-1 antibody and depletion of adherent monocytes inhibits IL-2 receptor expression (Lindemann, Kjeldsen and Cabret, 1995). It has been found that periodontitis patients have higher proportions of activated T cells locally, in diseased tissues, than in their peripheral blood (Okada, Kassai and Kida, 1984). The proportion of activated CD4 cells increases with disease severity and in sites showing recent AL. The number of T-helper cells increases only slightly, when the gingival lesion progresses from one showing mild inflammation to one exhibiting severe inflammation, but the number of activated cells quadruples (Reinhardt *et al.*, 1988b; Malberg *et al.*, 1992). The proportion of T-suppressor cells to activated T-suppressor cells has been demonstrated to remain relatively constant (Malberg *et al.*, 1992). However Reinhardt *et al.* (1988b) found a three-fold increase in the percentage of activated T-suppressor cells between healthy and active sites. The expression of IL-2 and HLA-DR has been found to be highest on T-helper cells and B cells isolated from crevicular fluid, intermediate on gingival tissue cells and lowest on peripheral blood lymphocytes (Takeuchi, Yoshie and Hara, 1991). These studies indicate that cells are activated locally.

1.2.5.3 T-helper cell subsets

Cytokines released by CD4 cells have been shown to determine the type of immune response, which occurs (Mosmann and Coffman, 1989). Activated CD4 cells in periodontal lesions have been shown to be capable of releasing various cytokines. The highest number of cytokine-producing cells has been found in the gingiva adjacent to the crevice where stimulation by antigens of plaque bacteria is most evident (Yamazaki, Nakajima and Hara, 1995). CD4 cells have been classified in the mouse according to their cytokine profiles into T-helper(Th)-1 and Th-2 subsets (Mosmann *et al.*, 1986; Romagnani, 1991). Th-1 and Th-2 cytokines may play an important role in determining the outcome of infection (Yamamura *et al.*, 1992; Mosmann and Sad, 1996). Th-1 cells secrete IL-2, IFNγ and tumour necrosis factor beta (TNFβ); Th-2 cells secrete IL-4, IL-5 IL-6, IL-10 and IL-13. Both subsets produce IL-3, tumour necrosis factor alpha (TNFα) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mosmann and Coffman, 1989; Kelso, 1995). In humans, Th-1 and Th-2 cells are not as clearly defined by their cytokine profiles and IL-2, IL-6, IL-10 and IL-13, are not restricted to a specific subset. Th-0 cells have also been identified which secrete IL-4 and IFNγ; they may possibly represent a precursor T-helper cell type (Modlin and Nutman, 1993). In addition, cells producing large amounts of the anti-inflammatory cytokine transforming growth factor-β have been named Th-3 (Mosmann and Sad, 1996). However, Kelso (1995) disagrees with the theory of distinct Th subsets and suggests that cytokine-producing cells cannot be separated into distinct phenotypes.

Mixed cytokine patterns of human CD4 cells have been demonstrated in some studies (Kelso, 1995; Mosmann and Sad, 1996). It appears likely, that the appropriate Th-1 or Th-2 response is determined by a complex interaction between multiple factors. Specific pathogens and varying levels of antigen dose may illicit different responses (Mosmann and Sad, 1996). In addition, it has been shown that APC may determine the type of T cell subset which is induced (Gemmell, Marshall and Seymour, 1997). The CD4 cell subsets Th-1, Th-2 and Th-0 have all been identified in gingival and periodontal lesions. More than one subset has been identified simultaneously (Wassenaar *et al.*, 1995; Gemmell and Seymour, 1998). Th-1 cytokines, IL-2 and

IFNγ enhance cell-mediated immunity while IL-4, produced by Th-2 cells, suppresses cell-mediated immunity and stimulates humoral immunity and B cell development (Mosmann and Coffman, 1989). Th-1 cells can provide some B cell help, but at higher T:B cell ratios this is suppressed (Romagnani, 1991).

The Th-1 cytokines IFNγ and IL-2 have been shown to be produced spontaneously by gingival mononuclear cells (GMC) extracted from diseased tissues (Seymour *et al.*, 1985). Pg can further stimulate IL-2 release from GMC of patients with EOP (Manhart *et al.*, 1994). Another study found cytokine production characteristic of a prominent Th-1 and a reduced Th-2 response in diseased GMC following culture and stimulation (Ebersole and Taubman, 1994). However, it has been demonstrated that peripheral blood mononuclear cell cultures stimulated with Pg or *Fusobacterium nucleatum* (Fn) show reduced IL-2 activity compared with unstimulated cell cultures (Gemmell and Seymour, 1994). In addition IFNγ could not be detected in cultures containing both bacteria. In GMC cultures, IFNγ was only detected at very low levels in 10 of 27 supernatants. Another study found no skewing of cytokines towards either a Th-1 or Th-2 profile in unstimulated GMC (Prabhu, Michalowicz and Mathur, 1996).

Cytokine production representative of Th-2 cells has been identified from memory T cells and GMC stimulated with periodontal bacteria from periodontal lesions (Manhart *et al.*, 1994; Yamazaki, Nakajima and Hara, 1995). Reports of Pg T cell lines demonstrated cytokine profiles representative of Th-1, Th-2 and Th-0 in both gingivitis and periodontitis lesions (Gemmell, Marshall and Seymour, 1997). Considering the high numbers of B cells and plasma cells in the advanced periodontal lesion, it is possible that Th-2 cells play a major role in the pathogenesis of chronic periodontitis (Mathur and Michalowicz, 1997). This theory is supported by two recent studies showing a predominant Th-2 response in periodontitis lesions (Tokoro *et al.*, 1997; Iwasaki *et al.*, 1998). In another report CD4 T cell clones active against type I collagen were isolated from a series of periodontitis tissue samples. Cytokines representative of Th-2 cells were expressed by eighty percent of these clones. In contrast T cell clones reactive to Pg, Aa and *Prevotella intermedia* (Pi) showed Th-0 type cytokine profiles. They produced IFNγ and IL-4. These results suggest that Th-2 CD4 cells may contribute to the periodontitis lesion in an autoimmune manner, but are

under the control of Th-0 clones which are stimulated by antigens of periodontal pathogens (Wassenaar *et al.*, 1995).

Gemmell and Seymour (1998) used an intracellular technique to determine cytokine profiles and found no differences in the numbers of IL-4 and IFNγ producing CD4 or CD8 cells, between gingivitis and periodontitis tissues. This study also demonstrated the presence of a higher percentage of CD30+ (a member of the TNF/nerve growth factor receptor family) cells, which were also CD4+ and CD8+, extracted from periodontitis lesions than from healthy/gingivitis lesions. CD30+ cells are found under normal conditions associated with B cell follicles of lymphoid tissue and germinal centres, rather than in the peripheral blood (Del Prete *et al.*, 1995). CD30 molecules are also more often expressed on Th-2 and Th-0 cells. It is possible that these cells are important in the local activation of B cells, which occurs in the periodontitis lesion (Gemmell and Seymour, 1991). Another recent study by the same group showed a predominantly protective Th-1 response in the spleens of BALB/c mice after immunisation with Pg outer membrane proteins (Gemmell *et al.*, 1998). In summary, studies of T-helper cells in periodontitis patients suggest that either no differentiation into distinct phenotypes exists, or that the range of subsets represents different stages of the disease.

1.2.5.4 B cells

As has already been stated above, B cell activation is considered to be an important feature of the chronic inflammatory periodontal lesion. B cells are under the control of helper T cells and therefore require close contact with them in order to be activated. The numbers of plasma cells have been found to correlate with CD4 cells. In addition, a tendency of plasma cells to form clusters near to T cells has also been noted (Tonetti, 1993).

It has recently been shown that T cell induced differentiation of B cells requires lymphocyte function-associated antigen-1 (LFA-1)/ICAM-1 and CD2/LFA-3 interaction (Gemmell, Sved and Seymour, 1995) (Figure 1.2). A high percentage of gingival B cells from both healthy/gingivitis and AP tissues have been found to express the adhesion molecules ICAM-1 and LFA-3, compared with peripheral blood

(50%:30%). In addition, gingival T cells from the same tissues have been found to express CD2 in a ratio of 50%:76% and LFA-1 in a ratio of 45%:45% with the peripheral blood. Stimulation of the gingival cells *in vitro* with Pg and Fn did not increase the percentage of CD2+ T cells and ICAM-1+ and LFA-3+ B cells, but did cause a rise in the number of T cells expressing LFA-1. The reverse situation occurred when peripheral blood cells were stimulated. These results indicate that gingival lymphocytes express adhesion molecules. They also suggest that these cells are already activated *in vivo* and that their phenotype is consistent with cell-cell contact occurring in the gingiva.

Intercellular adhesion used to be thought to precede antigen presentation. However, it has been reported that antigen binding induces the cell to express adhesion molecules, which augments the effect of other signals (Pardi, Inverardi and Bender, 1992). The increase in the percent of LFA-3 and ICAM-1 expressing B cells in both the healthy/gingivitis and AP tissues compared with peripheral blood, may indicate a rise in the numbers of antigen-specific cells which have already presented antigen to T cells. This supports previous studies that demonstrated local activation of B cells (Gemmell and Seymour, 1991). However two recent reports indicated the presence of pre-plasma cells in periodontitis sections, but no evidence of local proliferation of these cells. It was concluded that B-memory cells are recruited to periodontitis lesions from the lymph nodes where they differentiate into antibody-producing plasma cells (Takahashi, Lappin and Kinane, 1996; Kinane *et al.*, 1998; Lappin *et al.*, 1999).

The idea of polyclonal B cell activation has for a number of years been considered to be a possible mechanism for stimulation of the B cell response in periodontitis (Seymour, 1987; Tew, Engel and Mangan, 1989). This polyclonal B cell stimulation may be a feature of certain forms of periodontitis (Mallison *et al.*, 1988). It may on the other hand occur as a result of gingival penetration of LPS, in the presence of IL-2 (which can promote clonal expansion of B cells), thus augmenting specific B cell responses (Tew, Engel and Mangan, 1989). Alternatively, superantigenic stimulation of Vβ T cell families may occur in the inflamed tissues (Zadeh and Kreutzer, 1996) (Figure 1.2). The large quantities of antibodies produced are non-protective and wasteful of the host's antimicrobial responses.

1.2.5.5 Natural killer cells

Natural killer cells are large granular lymphocytes. They are cytotoxic to virally infected cells and kill by inducing apoptosis (Mathur and Michalowicz, 1997). They may contribute to the pathogenesis of periodontitis by a similar activity against fibroblasts and other host cells (Seymour *et al.*, 1984; Lindemann, 1988). The numbers of NK increase from health, where very few cells are present, to gingivitis, to periodontitis (Cobb *et al.*, 1989). However the percentage of the total lymphocyte population occupied by NK decreases from 19% in health to 6.6% in gingivitis and 7% in periodontitis. It is possible NK may have a regulatory roll in periodontitis since clusters of B cells or plasma cells have been found to have NK associated with them, which may influence their activity and proliferation (Wynne, Walsh and Seymour, 1986). NK have a greater inhibitory effect than suppressor T cells and therefore an imbalance in the NK to non-NK ratio may up-regulate the humoral response. This may bring about the conversion of a stable T cell to a destructive B cell lesion. The major signal for up-regulation of NK cytotoxic activity seems to be LPS of Gram negative periodontal pathogens (Lindemann, 1988). In addition NK may be subject to regulation by activating and inhibitory signals (Cobb *et al.*, 1989). NK activity is increased by IL-1 and may be down regulated by immune complexes and PGE_2 which suppress IL-2 production and indirectly influence NK (Seymour, 1987).

1.2.6 Summary

Progression from gingivitis to periodontitis, or from a quiescent state to an inflamed active state in periodontitis is dependent on the balance between the host defence system and the microbial challenge, at any one point in time. Numerous as yet undiscovered features of bacterial pathogenicity might allow the plaque microbiota to overcome the inflammatory or immune defence mechanisms. In this situation the host might respond in such a way that the bacterial challenge is contained and the balance restored. Alternatively, the host may be unable to respond effectively and disease progression might occur. It has been suggested that loss of attachment moves the tissue away from the margin of the subgingival plaque. This may arrest the disease

process until the plaque proliferates apically and again comes into close proximity to the lesion (Page *et al.*, 1997).

It appears from recent research that the immune response present in chronic periodontitis is initiated and activated locally in the gingival tissues. Evidence for this theory comes from studies of the resident tissue cells, present in normal healthy periodontium. Fibroblasts, junctional epithelial cells and vascular endothelial cells play an important role in the activation of the inflammatory response. They also have the ability to produce inflammatory mediators and matrix metalloproteinases in response to LPS and other bacterial products, and thus contribute to tissue damage (Takata *et al.*, 1988; Zappa *et al.*, 1992).

Lymphocytes and antibodies have been found in peripheral blood, which respond to periodontal bacteria (Ebersole and Taubman, 1994). It is possible that these cells and antibodies have escaped from the lesion into the systemic circulation. Alternatively, cross-reactivity may occur between periodontal pathogens and the normal gut flora. In this case, interpretation of systemic antibody titre and avidity levels, in patients and controls, should be made with caution. Future research in this area should help to identify which are the immunodominant antigens involved in the aetiology of chronic periodontitis.

The presence of Th-0, Th-1, Th-2 and naïve and resting and activated memory T cells in health, gingivitis and periodontitis may represent different stages in the disease process (Mathur and Michalowicz, 1997). Naïve Th-0 cells may be representative of an early periodontal lesion reacting with periodontal pathogens. Th-2 cell clones could perpetuate the chronic nature of the disease (Wassenaar *et al.*, 1995). However, separation into distinct T cell subsets may not exist. Therefore, investigating levels of specific cytokines in the gingival tissues, rather than concentrating on T cell clones, may be more relevant to furthering our understanding of the pathogenesis of periodontitis (Gemmell, Marshall and Seymour, 1997).

Immune susceptibility can influence the progression of periodontitis in two ways. Whether or not the lesion progresses rapidly resulting in marked destruction of connective tissue and bone may depend upon the quality of the humoral response. The

production of specific antibodies that eradicate pathogenic micro-organisms could halt the disease process. In contrast, if the antibodies produced are either ineffective at eliminating the bacteria or directed against the host tissues the destruction may progress (Wassenaar *et al.*, 1995; Mathur and Michalowicz, 1997). In addition, if B cells are activated polyclonally, an expansion of the B cell population would occur, secreting large amounts of IL-1 which would mediate tissue destruction via several pathways (Gemmell and Seymour, 1994; 1998).

1.3 The Role of Cytokines in the Inflammatory and Immune Response

1.3.1 Introduction

The etymon of "cytokine" derives from the Greek words kytos meaning vessel or hollow, and hence cell, and kinesis meaning movement. The term cytokine is therefore applied to molecules that move or transmit information between cells. Cytokines may be divided into two broad groups. These are the inflammatory or catabolic proteins and the reparatory or anabolic growth factors. The first group is further subdivided into:

1. The interleukins which carry information between leucocytes.
2. The chemokines (chemotactic cytokines) which are involved in cell recruitment.
3. The interferons which influence lymphoctye activity (Offenbacher, 1996).

1.3.2 General Characteristics of Cytokines

Cytokines are small proteins with molecular weights between 5 and 70 kDa (Bendtzen, 1994). Many different cell types secrete cytokines in response to injury or infection. These proteins are secreted and are biologically active in very small amounts (in the range of picomolar to femtomolar). Most cytokines have been named according to a single biological property. However, the majority are multifunctional molecules with various biological actions and target cells. Generally their most important functions appear to be accomplished locally (Whicher and Evans, 1990). Cytokines exert their

action by binding to specific receptors on the surface of effector cells. This stimulates intracellular activation of the cell, which modifies behaviour and leads to alterations in biological outcome. Signals transmitted between adjacent but different cell types are known as paracrine. Autocrine signals occur between similar cell types or on the same cell. In some situations where receptors become saturated, cytokines such as TNF, IL-1 and IL-6 may spill over into the systemic circulation and stimulate distant tissues and organs (Offenbacher, 1996).

1.3.2.1 Cytokine function

Many cytokines have overlapping functions and receptor binding specificities. They form a complex network which controls the immune response (Yamazaki *et al.*, 1997). They may act synergistically or may inhibit the action of another cytokine (Kjeldsen, Holmstrup and Bendtzen, 1993). The concentration of a cytokine affects its functions (Page *et al.*, 1997). A cytokine may have different forms of receptors on different cell types or on the same cell type, which may allow completely opposing biological outcomes. In addition, raised levels of the proinflammatory cytokines may be evident during periods of healing, as well as during bursts of destruction. It is important to bear these points in mind when interpreting results of *in vitro* experiments with single cytokines or simple combinations of cytokines. Results of experiments such as these may not mirror the true situation in the living organism (Whicher and Evans, 1990). Furthermore, association of a specific cytokine with a disease entity does not imply causation.

1.3.2.2 Regulation of cytokines

Cytokine regulation is achieved through several mechanisms. Control of cytokines takes place at the gene activation level, during secretion and circulation, and at the target T cell receptor (TCR) level (Bendtzen, 1994). Cytokine production is usually short-lived and self-limiting (Howells, 1995). In addition, many cytokine receptors exist in soluble forms, which may be cleaved from the target cells and bind and possibly neutralise cytokines in the extracellular environment (Bendtzen, 1994). The local environment may influence the level of expression of cell receptors. For

example, platelet-derived growth factor (PDGF) usually triggers fibroblasts to increase the quantity of extracellular matrix they produce. In areas of inflammation however, IL-1 will stimulate fibroblasts to secrete PGE_2 and PDGF receptors are down-regulated. Down-regulation of PDGF receptors causes the fibroblasts to be unresponsive to PDGF. In other words a catabolic response replaces an anabolic one (Offenbacher, 1996). Therefore the circumstances under which a particular cell is living at any one time influence the regulation of the cell by cytokines. Proinflammatory cytokines are also regulated by high affinity autoantibodies and anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1ra), IL-4 and IL-10 (Bendtzen, 1994).

1.3.3 Proinflammatory Cytokines

The most important catabolic cytokines are IL-1, IL-6 and TNF. Interleukin-1 and TNF will be discussed in more detail here, together with IL-1ra and IL-10. Work reported in this thesis includes analysis of polymorphisms in the *TNF* and *IL10* gene clusters. A discussion of IL-1 is included because of the similarity and overlap in biological function between this cytokine and TNF. IL-10 is included in the proinflammatory cytokines, despite serving to dampen many of the destructive aspects of the immune response. IL-1ra another anti-inflammatory cytokine will be covered in the sections on IL-1.

1.3.3.1 Interleukin-1

IL-1 exists in two forms IL-1α and IL-1β which share only 26% amino acid sequence homology despite binding to the same type of high affinity receptor (Whicher and Evans, 1990). Separate genes located on chromosome two code for each form. Both forms are produced as 31 kDa precursors, which undergo enzymatic cleavage to the active 17.5 kDa proteins (Tatakis, 1993). IL-1 is mainly secreted by monocytes or macrophages but can also be secreted by most nucleated cells (Jandinski *et al.*, 1991; Matsuki, Yamamoto and Hara, 1991; 1992).

Bacterial lipopolysaccharide is a potent stimulator of IL-1 production (Offenbacher, 1996). IL-1 can modulate the biological activity of a wide range of cells and is

autostimulatory in order to increase the production of IL-1. The most important function of IL-1 is to induce the expression of other cytokines. The first mode of action of IL-1 to be recognised was its ability to cause fever. It was known as endogenous pyrogen (Atkins, 1960). In addition, IL-1 has been found, together with TNF and IL-6, to induce hepatic acute phase protein synthesis (Whicher and Evans, 1990), upregulate T and B lymphocyte responses (Offenbacher, 1996; Gemmell and Seymour, 1998), stimulate monocytes to release PGE_2, instigate bone resorption by osteoclasts (Gowen, Nedwin and Mundy, 1986) and inhibit bone formation (Stashenko *et al.*, 1987b).

The discovery of an antagonist of IL-1 activity had important implications for the role of IL-1 in disease (Bendtzen, 1994). IL-1ra modulates the activity of IL-1. It is an inhibitor protein which can bind to the IL-1 receptor (IL-1R) without activating the cell. Monocytes and PMN produce IL-1ra and keratinocytes produce an intracellular form of the antagonist (Gemmell, Marshall and Seymour, 1997). Steroids, IL-4 and IL-10 induce IL-1ra synthesis (Tatakis, 1993). IL-1 receptors exist in four forms. These are the two membrane bound receptors IL-1RI and IL-1RII and the two soluble forms sIL-1RI and sIL-1RII. IL-1RI are found on T cells, fibroblasts, hepatocytes, endothelial and epithelial cells whereas IL-1RII are found on B cells, macrophages and neutrophils (Tatakis, 1993).

All three members of the IL-1 group (IL-1α, IL1-β and IL-1ra) bind to IL1-RI and IL1-RII. However, since IL1-RII has a very short cytoplasmic tail most of the signalling takes place through IL-1RI. In addition, sIL-1RI only binds IL-1ra and sIL-1RII binds IL-1β as effectively as the membrane-bound form and IL-1ra 2000 times less so (Symons, Young and Duff, 1995). Soluble IL-1RII also binds the IL-1β precursor (which cannot activate the type I membrane-bound IL-1R) thereby preventing it from being cleaved to the active 17 kDa form. Therefore sIL-1RII works together with IL-1ra to control the bioactivity of IL-1β. Corticosteroids induce IL-1RII mRNA synthesis and the release of sIL-1RII, mostly from neutrophils. This may partly explain their anti-inflammatory and immunosuppressive actions (Bendtzen, 1994).

1.3.3.2 IL-1 in relation to periodontal disease

In the periodontium IL-1β is the predominant form over IL-1α and is present at higher levels in active diseased sites, compared with healthy or stable diseased sites (Hönig *et al.*, 1989; Jandinski *et al.*, 1991; Stashenko *et al.*, 1991b; Chen *et al.*, 1997). The variation in IL-1 concentrations between sites in the same individual, and the lack of variation in serum IL-1β levels between AP patients and healthy controls, points towards localised rather than systemic production of IL-1 (Tatakis, 1993; Chen *et al.*, 1997). Activated keratinocytes and Langerhans cells, which are derived from monocytes and found within the gingival tissues, produce IL-1α and IL-1β (Hillmann, Hillmann and Geurtsen, 1995). Macrophages, PMN, B cells and fibroblasts secrete IL-1β (Jandinski *et al.*, 1991; Takahashi, Poole and Kinane, 1995; Hendley, Steed and Galbraith, 1995; Takiguchi *et al.*, 1997; Gemmell and Seymour, 1998). IL-1ra has been detected in GCF from patients with periodontitis (Howells, 1995; Kabashima *et al.*, 1996). In addition, monocytes and macrophages in the tissues were found to stain positive for IL-1ra.

It has been shown that a number of periodontopathic bacteria and their products can stimulate the production of IL-1 by host cells (Bom-van Noorloos *et al.*, 1990; Takada *et al.*, 1991; Gemmell and Seymour, 1993; 1998; Roberts, Richardson and Michalek, 1997). Levels of IL-1β correlate with the numbers of infiltrating macrophages and other leucocytes (Tokoro, Yamamoto and Hara, 1996); the quantitiy of GCF IL-1β correlates with periodontal status (Preiss and Meyle, 1994; Hou, Liu and Rossomando, 1995; Liu *et al.*, 1996). IL-1 stimulates fibroblasts, endothelial cells, monocytes and granulocytes resident in the gingival connective tissue to express adhesion molecules (Takahashi *et al.*, 1994; Ozaki *et al.*, 1996). As discussed earlier, these adhesion molecules aid the passage of immune response cells from the capillaries into the inflamed tissues by chemotaxis. In addition, it has been suggested that lymphocytes and monocytes attach to fibroblasts and become resident in the inflamed connective tissue (Hayashi *et al.*, 1994; Takahashi *et al.*, 1994).

IL-1 is a key mediator in inflammation and tissue destruction in periodontal disease (Assuma *et al.*, 1998). Both IL-1 and TNFα strongly induce MMP expression in

resident and immigrant cells, but IL-1 is far more potent than TNFα (Birkedal-Hansen, 1993; van der Zee, Everts and Beertsen, 1997). MMP degrade the extracellular matrix. It has been demonstrated that IL-1 can induce the production of plasminogen activator, IL-6 and PGE_2 as well as MMP in human gingival fibroblasts (Richards and Rutherford, 1988; Mochan, Armor and Sporer, 1988; Bartold and Haynes, 1991). IL-1 has also been shown to increase the number of IL-1RI on gingival fibroblasts. This was suggested to be a method of amplifying gingival inflammation (Kanda-Nakamura, Izumi and Sueda, 1996). IL-1 triggers procollagenase, stromelysin and prostaglandin synthesis in periodontal ligament cells (Richards and Rutherford, 1988; Tatakis, 1993; Nakaya *et al.*, 1997). In addition, IL-1 stimulates osteoclasts to resorb bone (Horton *et al.*, 1972; Dewhirst *et al.*, 1985) and inhibits the formation of bone (Stashenko *et al.*, 1987b). More specifically, IL-1 mediated resorption of bone has been shown to be dependent on stimulation by periodontopathogens (Bom-van Noorloos *et al.*, 1990; Ishihara *et al.*, 1991).

Prostaglandin E_2 causes increased vascular dilation and permeability. It also stimulates macrophages to secrete MMP and has been shown to trigger bone resorption *in vitro* (Birkedal-Hansen, 1993), thus acting synergistically with IL-1 and TNFα. IL-1 has been shown to stimulate gingival antigen-presenting cells to express HLA class II proteins in order to aid antigen recognition by T cells and B cells (Tatakis, 1993). It also upregulates receptors for complement and immunoglobulin on monocytes and neutrophils (Offenbacher, 1996).

A recent study used a quantitative method to measure cytokine mRNA levels in healthy and diseased gingival sections from the same AP patients. The samples were also assessed for macrophage and T cell markers using immunohistochemistry and for mRNA expression of cytokines, using *in situ* hybridisation (Roberts *et al.*, 1997). The results showed that higher levels of both IL-1β and IL-1ra were present in inflamed tissues than in healthy tissues. The levels of the two cytokines were also significantly correlated. In the diseased sections intense staining for IL-1β and IL-1ra was evident in the epithelium. In addition, there was some positive subepithelial staining of mononuclear cells for IL-1ra mRNA. These results provide evidence of a protective response by macrophages and epithelial cells to antigens produced by plaque bacteria.

1.3.3.3 Tumour necrosis factor

The TNF family consists of three members (Eigler *et al.*, 1997); TNFα, formally known as cachectin, TNFβ, also referred to as lymphotoxin alpha, and lymphotoxin beta. TNFα was originally described by its ability to cause *in vitro* and *in vivo* tumour necrosis (Carswell *et al.*, 1975). Ten years later the role of cachectin in infections was investigated (Beutler *et al.*, 1985). Amino acid sequencing and cDNA cloning showed the genes for these two factors to yield an identical 17 kDa protein (Whicher and Evans, 1990). TNFα in the same manner as IL-1 is secreted as a precursor protein, with a molecular weight of 26 kDa, which is cleaved to yield the active form. TNFα was also found to share 28% homology, some biological functions and a common receptor with lymphotoxin alpha. The genes encoding the TNF family lie within the class III *HLA* complex situated on the short arm of chromosome 6. TNFα is mainly secreted by macrophages, and TNFβ by T cells. In areas of inflammation TNFα is more important but both forms have the same effects (Whicher and Evans, 1990). Nowadays TNFα is often referred to as TNF.

TNF and IL-1 are produced in response to the same stimuli and, other than B cell proliferation and T cell production of IL-2 (upregulated by IL-1), show similar biological effects (Whicher and Evans, 1990). Levels of TNF and IL-1 are often highly correlated. At low levels these cytokines stimulate the expression of class II HLA antigens, adhesion molecules and Fc gamma receptors (FcγR), thus assisting in microbial clearance (Offenbacher, 1996). At higher levels, as already discussed above, they cause connective tissue destruction and bone resorption. On its own TNF is a 1000-fold less potent stimulator of bone resorption than IL-1β (Stashenko *et al.*, 1987a). However synergy between IL-1α or IL-1β and TNF results in a two-fold increase in IL-1 activity and a 100-fold increase in TNF activity.

TNF receptors exist in membrane bound and soluble forms in the same manner as IL-1 receptors (Bendtzen, 1994). TNF-RI and TNF-RII, the membrane bound receptors, bind TNFα and TNFβ with equal affinity. However, the soluble forms (sTNF-RI and sTNF-RII) bind TNFα more effectively. They may serve to regulate the activity of TNFα, which is a more potent inflammatory cytokine than TNFβ.

1.3.3.4 TNF in relation to periodontal disease

TNFα, IL-1α and IL-1β have been identified in periodontitis lesions from patients with AP (Stashenko *et al.*, 1991a; Matsuki, Yamamoto and Hara, 1992). Levels of TNFα and IL-1 were significantly higher in diseased than healthy sites. However, cells containing IL-1β were found in much greater numbers than those producing TNFα and IL-1α (Stashenko *et al.*, 1991a). A recent study examined IL-1β and TNFα production in oral PMN culture supernatants of 40 AP patients and 40 controls. Unstimulated oral PMN released significant amounts of both cytokines, but there were no differences between patients and controls. The authors noted that there was marked variability in TNFα production between individuals (Galbraith *et al.*, 1997). Another group found negligible mRNA TNFα expression in the gingival tissues of periodontitis patients (Tokoro, Yamamoto and Hara, 1996).

Recently, Roberts *et al.* (1997) showed statistically significant differences in TNFα mRNA expression between diseased and healthy sections from the same AP patient. Macrophages and T lymphocytes were found to express TNFα mRNA using immunohistochemistry and *in situ* hybridization. This study provides convincing evidence of a role for TNFα in periodontal destruction. It employed *in situ* hybridisation, which has a higher threshold for detection of mRNA expression and only stains cells expressing increased amounts. In addition, by using patients as their own controls individual variability was excluded. The presence of low concentrations of TNFα in GCF has been demonstrated in both AP and EOP patients (Rossomando, Kennedy and Hadjmichael, 1990; Yavuzyilmaz *et al.*, 1995). In both studies levels of the cytokine showed a weak negative correlation with mean pocket depths and gingival index scores. Howells (1995) also found that levels of TNFα in GCF were below detection when employing sensitive ELISA techniques. However levels of TNFα in the gingival tissues taken from the same sites were in the 200 pg μl^{-} range.

The biological activities of IL-1 and TNF, which may be important in the pathogenesis of periodontal disease, have been discussed above. It has been demonstrated that IL-1β and TNFα can induce the expression of MCP-1 in human periodontal ligament fibroblasts. These cytokines also act synergistically to stimulate MCP-1 gene expression (Ozaki *et al.*, 1996). Gingival fibroblasts produce collagenase

and degrade type I collagen, resulting in connective tissue destruction, when stimulated by TNF (Meikle *et al.*, 1989). In addition, periodontal ligament cells treated with TNFα show enhanced production of PGE_2 and bone resorption in mouse calvaria (Saito *et al.*, 1990).

When all the above findings are taken together, they appear to suggest that increased levels of TNFα in periodontal lesions are representative of an established inflammatory and immune response. Salvi and co-workers (1998) have examined GCF IL-1β, TNFα and PGE_2 secretion patterns in type I diabetes mellitus patients with periodontal disease. They found that diabetics had significantly increased levels of GCF, PGE_2 and IL-1β compared with non-diabetic controls with similar periodontal status. In addition diabetics with moderate to severe disease (group B) had almost two-fold higher levels of these mediators compared with diabetics with gingivitis or mild periodontitis (group A). However, TNFα GCF levels in diabetics were barely detectable and no differences were found between group A and group B. Low levels of TNFα in GCF from patients with periodontitis, and high levels in the tissues at disease active sites, may indicate loss of the chemotactic gradient with increasing inflammation. Monocytes and neutrophils retained in the tissues would then trigger destruction via a number of pathways. Alternatively, increased levels of the soluble forms of TNF receptor in the GCF of patients with periodontal disease might serve to mop up excess TNFα (Howells, 1995).

1.3.3.5 Interleukin-10

The gene encoding IL-10 is situated on chromosome one. It yields a protein with a molecular weight of 19 kDa. Macrophages, T cells (across all the various subsets), B cells, mast cells and keratinocytes can all produce IL-10 after activation (Bendtzen, 1994; Gemmell, Marshall and Seymour, 1997).

A growing body of evidence implicates IL-10 as a key player in regulating the immune and inflammatory response. It has often been described as an anti-inflammatory cytokine (Isomäki *et al.*, 1996). Its main function is to suppress monocyte/macrophage activity by counteracting the stimulatory effect of IFNγ, IL-1β and TNFα (De Waal Malefyt *et al.*, 1991). TNFα secreted by macrophages stimulates the release of IL-10

from the same cells, thereby suppressing TNF production in an autocrine manner (Platzer *et al.*, 1995). IL-10 has also been found to reduce IL-1, IL-6 and IL-8 synthesis by monocytes and neutrophils and to prolong the expression of IL-1ra (De Waal Malefyt *et al.*, 1991; Gemmell, Marshall and Seymour, 1997). IL-10 inhibits HLA class II and B7 expression by macrophages (Ding *et al.*, 1993; Chadban *et al.*, 1998). B7 is an important co-stimulatory molecule for macrophage/T cell antigen presentation. IL-10 therefore abrogates antigen presentation by macrophages and indirectly inhibits T cell activation (Bendtzen, 1994). Another indirect effect of IL-10 is through decreased production of IL-12 by macrophages, resulting in suppression of NK activity (Bendtzen, 1994). IL-10 production may also be down-regulated in a negative autoregulatory manner (Knolle *et al.*, 1998). It was suggested that this effect might allow the re-emergence of a state of functional responsiveness towards new proinflammatory stimuli.

IL-10 also acts directly on Th-1 and Th-2 cells to down-regulate the production of other cytokines. However, it appears that Th-1 responses are most affected (De Waal Malefyt, Yssel and de Vries, 1993; Bendtzen, 1994). Recently IL-10 has been shown to suppress T cell activation, matrix metalloproteinase production and extracellular matrix damage in human gut mucosa infections (Pender *et al.*, 1998). It also appears to suppress the production of cyclooxygenase-2 (COX-2), the inducible form of prostaglandin H synthase by down-regulating transcription (Mertz *et al.*, 1994). This in turn reduces PGE_2 production, which inhibits MMP sythesis. Parks *et al.* (1998) have demonstrated that IL-10 can differentially affect chemokine production of monocytes and human umbilical vein endothelial cells. They suggested that IL-10 might therefore influence the type of leucocytes that are recruited to the tissues at different stages of the inflammatory response. IL-10 itself has been shown to act as a chemotactic factor for CD8 cells; in addition, it may inhibit IL-8-induced movement of CD4 cells (Jinquan *et al.*, 1993).

Another important function of IL-10 is the regulation of proliferation and differentiation of activated B cells (Rousset *et al.*, 1992; Itoh and Hirohata, 1995). The effects of IL-10 are dependent on the state of activation of B cells after antigen binding. If IL-10 is present during initial activation it promotes apoptosis of B cells.

However IL-10 may prolong viability and support differentiation of preactivated B cells (Bendtzen, 1994; Itoh and Hirohata, 1995). This cytokine may therefore serve to amplify the humoral immune response (Rousset *et al.*, 1992). In a number of autoimmune inflammatory conditions, such as rheumatoid arthritis (RA) and systemic lupus erythematosus, raised IL-10 levels have been noted (Llorente *et al.*, 1994). IL-10 has been found to trigger the production of rheumatoid factor (Perez, Orte and Brieva, 1995) and other autoantibodies (Llorente *et al.*, 1995). IL-10 usually plays an anti-inflammatory role in response to infection and in a number of autoimmune diseases. However, this cytokine may on occasions enhance inflammation through the stimulation of B cell clones producing excessive amounts of autoantibodies.

1.3.3.6 IL-10 in relation to periodontal disease

It has been suggested that IL-10 may play an important role in the progression of periodontal disease. Gemmell *et al.* (1995) found that AP T cell lines from both peripheral blood and gingival tissue secreted IL-10 but clones derived from the peripheral blood of an individual with gingivitis did not. It has also been suggested by Stein and co-workers that IL-10 may contribute to auto-immune reactions against gingival tissues (Stein and Hendrix, 1996; Stein *et al.*, 1997). They demonstrated that GMC from AP lesions produce more IL-10 than cells from healthy tissue (Stein and Hendrix, 1996). IL-10 is able to favour the development of a particular clone of B cells (CD5), which can secrete high levels of autoantibody and is found in increased numbers in inflamed gingival sections (Sugawara *et al.*, 1992). Antibodies directed against IL-10 reduced the presence of cells secreting anti-collagen immunoglobulins by 80% (Stein and Hendrix, 1996). A further study of insulin-dependent (type I) diabetics investigated the response of PBMC to Pg LPS. In a subgroup of the study population, IL-10 production and the number of CD5 B cells was significantly increased compared to controls. A higher percentage of anti-collagen secreting cells was also observed. The authors suggested that a subset of type I diabetics may be more susceptible to developing periodontitis, through an autoimmune type of reaction directed against gingival connective tissue, if exposed to periodontal pathogens (Stein *et al.*, 1997).

Fujihashi *et al.* (1996) supported these findings in AP patients. They investigated cytokine profiles of CD4 cells extracted from inflamed periodontal lesions. Two distinct patterns emerged which were divided according to the presence or absence of mRNA for IL-10. When PBMC from the same patients were stimulated with concanavalin A, no distinct patterns were evident. These findings again support a localised defect in the immune response in periodontal disease.

More recently, another hypothesis for the role of IL-10 in periodontal disease has been proposed. Gemmell and Seymour (1998) demonstrated a significantly reduced number of IL-10+ CD8 cells from periodontitis lesions (AP) than from healthy/gingivitis (H/G) sections ($p = 0.033$). They suggested that in gingivitis IL-10 might suppress inflammation by decreasing macrophage activity, thereby preventing progression to periodontitis. No differences were found in the percentage of IL-10+ CD4 cells between the two disease categories. However, some individuals were found to have high numbers of IL10+ T cells, and others demonstrated low levels or none, regardless of disease category. It appears that the overall variation between the AP and H/G groups in IL-10+ CD8 cells may have been due to the distribution of individual IL-10 secretion patterns, rather than due to differences in pathogenesis between the two disease categories. Further studies should help to elucidate the possible role of IL-10 in periodontal disease.

1.3.4 Summary

The cytokine network plays a major role in immune homeostasis. Complex synergistic and inhibitory interactions exist between the various members. This allows for redundancy within the system without devastating effects to the host. IL-1 and TNF are proinflammatory cytokines. At low levels they activate the host defence mechanisms in a protective manner and at higher levels they cause collagen breakdown and bone destruction. The effects of these mediators are modulated by soluble cytokine receptors, steroids, autoantibodies to cytokines and by anti-inflammatory cytokines such as IL-1ra and IL-10 (Bendtzen, 1994).

There is sufficient evidence to implicate IL-1 and TNF in progressive periodontitis, but the role of IL-10 is at present less certain. Despite the anti-inflammatory nature of IL-

There is sufficient evidence to implicate IL-1 and TNF in progressive periodontitis, but the role of IL-10 is at present less certain. Despite the anti-inflammatory nature of IL-10, it is possible that in some periodontitis patients IL-10 may contribute to the disease process by stimulating the production of autoantibodies against host tissues (Stein and Hendrix, 1996; Stein *et al.*, 1997). These findings require corroboration.

1.4 The Major Histocompatibility Complex

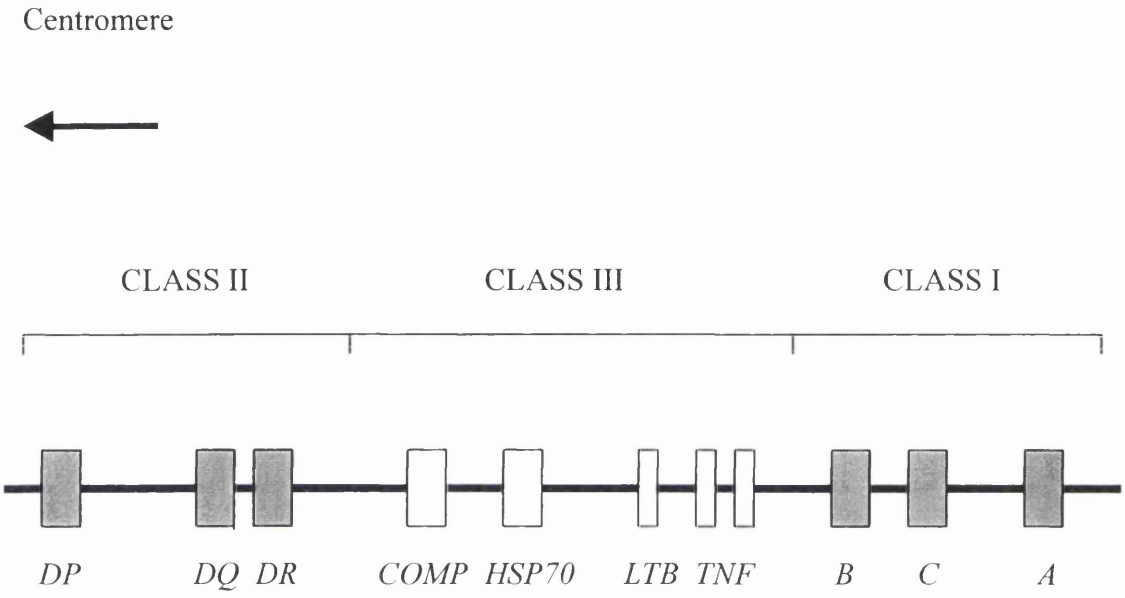
1.4.1 Introduction

The human major histocompatibility complex (*MHC*) is a large collection of multiple genes situated on the short arm of chromosome six (Figure 1.1). It covers an area of about 3800 kilobases of DNA (Schwartz, Felser and Mayr, 1995). The human *MHC* is also known as the human leucocyte antigen complex. The *HLA* complex is divided into three clusters of genes; classes I, II and III (Figure 1.1). Classes I and II loci code predominantly for cell surface antigens which are important for recognition of self and non-self peptides by T cells (Bodmer, 1995). A locus is the position on a chromosome at which a gene is found. Class III loci are situated between class I and class II and code for certain complement proteins (Meo *et al.*, 1977), heat shock protein 70 (Sargent *et al.*, 1989) and for the cytokines TNFα and TNFβ (Spies *et al.*, 1986). In addition, several other genes are present in this region.

1.4.2 The *HLA* Complex and the Immune Response

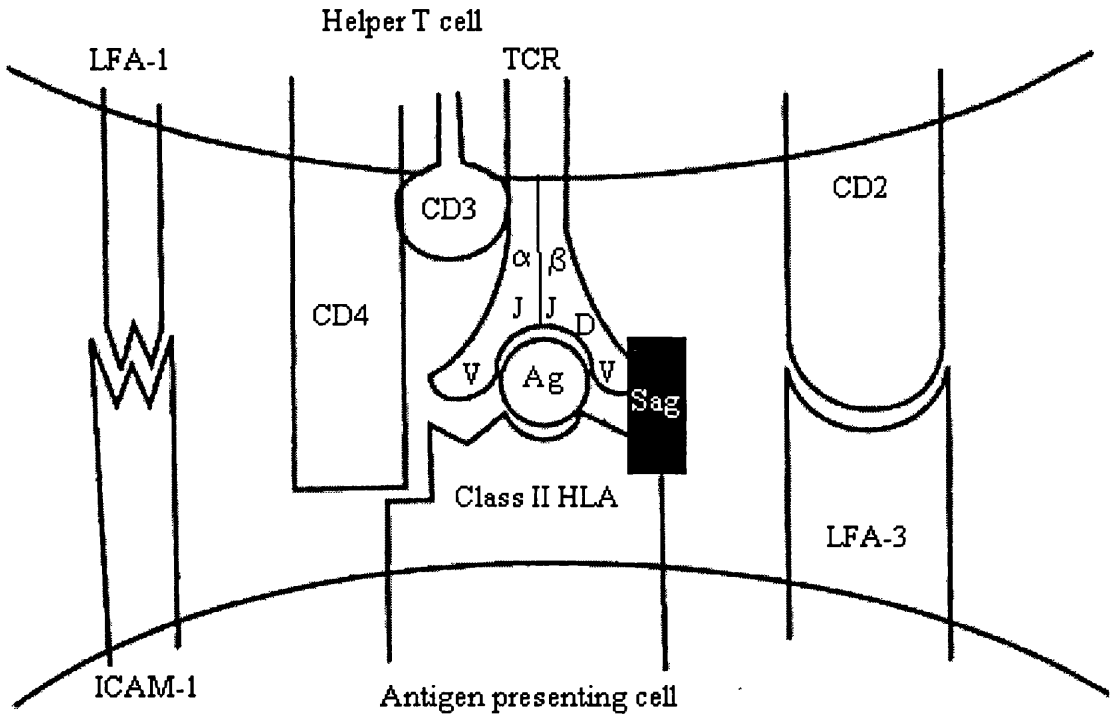
The cell surface molecules of the *HLA* complex and the T cell receptor (TCR) play an important role in immune responsiveness (Baines and Ebringer, 1992; Bodmer, 1995) (Figure 1.2). The TCR is a membrane bound molecule composed of α and β (or γ and δ) disulphide-linked chains. Each chain has a constant (C) and a variable region (V) and a joining domain (J). The β chain also has a diversity region (D). Numerous C, V, D and J genes exist, which are randomly rearranged to code for unique TCR molecules (Zadeh and Kreutzer, 1996). The variation in the V, D and J regions in the β chain, and the V and J regions in the α chain, determine the specificity of the antigen-binding

Figure 1.1 Diagrammatic representation of the human major histocompatibility complex showing the class I, II and III regions.



DP, DQ, DR = *HLA* class II loci, *COMP* = complement genes, *HSP70* = heat shock protein 70 locus, *LTB* = lymphotoxin β gene, *TNF* = tumour necrosis factor loci, *B, C* and *A* = *HLA* class I loci. Adapted from Wilson *et al.* (1995) and Rink and Kirchner (1996).

Figure 1.2 **Diagrammatic representation of antigen presentation to T helper cells.**



Adapted from Kinane and Davies (1990). See text for explanation of T cell receptor structure. TCR = T cell receptor, HLA = human leucocyte antigen, CD4 = cluster of differentiation antigen for T-helper cells, CD3 = signal transducing unit for the TCR, Ag = antigen, Sag = superantigen.

activation (Engel, 1996). The CD3 molecule transduces the signal received by the TCR to the inside of the cell (Roitt, 1994).

The *HLA* complex is amongst the most polymorphic regions of the human genome with each locus represented by multiple alleles (Bodmer, 1995). An allele is one of a number of possible alternative forms of a gene at a given locus. Each allele has a different sequence of DNA, which may code for different amino acids and thereby affect the structure and possibly the function of a protein product. It is assumed that the high degree of polymorphism displayed by the *HLA* complex was developed in order to protect the species from infection (Schwaiger and Epplen, 1995; Bodmer, 1995). Associations between particular HLA antigens and several diseases thought to have an autoimmune background, such as diabetes and RA, have been reported (Baines and Ebringer, 1992). More recently the relevance of HLA variation in influencing susceptibility to infection has also been determined (Hill, 1992).

1.4.3 Antigen Presentation

HLA molecules contain specialised binding sites for antigen peptides (Bjorkman *et al.*, 1987; Brown *et al.*, 1993; Stern *et al.*, 1994). These peptides are the products of antigen processing within the APC. Class I HLA antigens are found on the surfaces of most eukaryotic cells and are involved in presentation of self-antigens. They also present antigens derived from pathogens which invade cells, such as viruses. Class II HLA antigens are preferentially found on professional APC such as macrophages, B cells, dendritic cells and Langerhans cells. Other cell types may be stimulated to express class II antigens by the cytokine IFNγ (Pober *et al.*, 1983; Walsh, Seymour and Powell, 1986). These antigens present peptides derived from pathogens existing extracellularly such as most species of bacteria. The class I antigen peptide complex is presented at the cell surface to cytotoxic T cells. The class II antigen peptide complex is presented to helper T cells.

1.4.4 The Possible Role of HLA Antigens in Periodontal Disease

Particular class II *HLA* genes have been associated with protection from infectious diseases such as malaria (Hill *et al.*, 1991). Class II HLA antigens (DQ, DP and DR) have been identified on gingival Langerhans cells and have been shown to stimulate lymphocytes (Walsh, Seymour and Powell, 1987; 1990). Keratinocytes have also been shown to express HLA-DR but not –DQ (Walsh, Seymour and Powell, 1986). Langerhans cells correlate with the presence of Aa and Pg on the oral epithelium in periodontitis (Saglie *et al.*, 1987) and numbers of Langerhans cells have been shown to increase in gingival inflammation (DiFranco *et al.*, 1985; Seymour *et al.*, 1988; Günhan *et al.*, 1996). LPS from the periodontal pathogen Fn induces differential expression of HLA-DR and -DQ on Langerhans cells *in vitro* (Walsh, Seymour and Powell, 1987). It is possible that the humoral response to specific periodontal pathogens is under the influence of *HLA* genes. Work in our laboratories has indicated a wide variation in serum antibody titre and avidity to Pg and Aa within groups of periodontitis patients pre- and post-treatment (Mooney *et al.*, 1995). These varying responses could be associated with different *HLA* genotypes within a homogeneous population.

Molecular genetic techniques are now available to investigate in detail genetic polymorphisms such as those demonstrated by the *HLA* gene cluster (Schwartz, Felser and Mayr, 1995; Wade, 1996). A recent study in a population of Japanese patients demonstrated a significant association between an atypical *Bam*HI restriction site found in the *HLADQB* gene and EOP (Takashiba *et al.*, 1994; Ohyama *et al.*, 1996). In addition a large-scale linkage study of 100 families with EOP carried out by Wang *et al.* (1996) identified linkage between susceptibility to EOP and the *HLA* region of chromosome six (Hart and Kornman, 1997) (section 1.8.4.4).

1.4.5 Summary

Associations between the *HLA* complex and a number of chronic diseases have been established. In addition, its role in influencing susceptibility to infection has more

recently been uncovered. It appears likely that variation in *HLA* genotype between individuals also determines response to infection in periodontal disease.

1.5 Classification of Periodontitis

1.5.1 Introduction

The classification of periodontitis has evolved over the years in an attempt to parallel developments in diagnosis and increased knowledge of the aetiology and pathogenesis of the disease process. A number of different authors have proposed varying classifications (Page and Schroeder, 1982; Grant, Stern and Listgarten, 1988; Proceedings of the world workshop in clinical periodontics, 1989; Genco, 1990; Ranney, 1993; Carranza, 1996; Jenkins and Allan, 1999). Some of these have included gingivitis as well as periodontitis. The contents have ranged from a few distinct categories to a more complex layout. The classification presented in this thesis will include only inflammatory diseases of the supporting structures of the teeth, which have an underlying infectious aetiology (Table 1.1).

Periodontitis is thought to be a heterogeneous group of diseases with many similarities in microbial aetiology, pathogenesis and clinical presentation. The characteristics, which distinguish the different categories, are the age of onset and the extent and severity of the disease. However, there is considerable overlap between the disease categories.

1.5.2 Measurement of Disease Status

Diagnosis of periodontal disease is based on clinical measurement of attachment level and radiographic analysis. Severity of diseased is referred to in terms of AL and loss of alveolar bone height. The latter may be described as a proportion of the optimum bone height, or by direct millimetric measurement.

Table 1.1 Classification of periodontal disease.

Early-onset periodontitis

Prepubertal periodontitis (PP)

Localised PP

Generalised PP

Localised early-onset periodontitis (LEOP)

Generalised early-onset periodontitis (GEOP)

Incidental early-onset periodontitis (IEOP)

Adult periodontitis (AP)

Refractory periodontitis (RP)

Necrotising ulcerative periodontitis (NUP)

Periodontitis associated with systemic disease

1.5.2.1 Attachment level and probing pocket depth

Attachment level can be assessed using a graduated probe, by measuring the distance in millimetres from the amelocemental junction (ACJ) to the base of the probable periodontal pocket. Pocket depth is measured from the gingival margin to the base of the pocket. Pocket depth will only coincide with measurement of attachment level when the gingival margin is level with the ACJ. In situations where swelling of the gingivae has occurred causing "false pocketing", the attachment level measurement will be less than the pocket depth. The reverse will be the case when the gingival margin has receded apically. Recession is measured apically from the ACJ to the gingival margin.

Measurement of attachment level or pocket depth is not a true representation of the level at which the most apical cells of the JE lie (Nyman and Lindhe, 1997). Listgarten (1980) suggested distinguishing between the histological and clinical pocket depth, or in other words the actual depth and the measurement recorded by the probe. There are a number of factors which influence the measurement of attachment level. These include the thickness of the probe; the position of the probe in relation to the anatomical morphology of the tooth; the pressure applied during probing; and the degree of inflammatory cell infiltration of the tissues (Listgarten, 1980; Zappa *et al.*, 1991). Errors in recording probing pocket depth and attachment level can be reduced by using a suitable probe and by correct angulation of the probe. Variations in measurements due to probing pressure and the degree of inflammation of the tissues are more difficult to control. Pressure sensitive probes have been developed which enable the examiner to probe with a constant pressure (van der Velden and de Vries, 1978; Polson *et al.*, 1980). However these electronic probes are also subject to measurement errors (Robinson and Vitek, 1979; Polson *et al.*, 1980).

Recording of attachment level and probing pocket depth will be overestimated at inflamed sites and underestimated at sites which have healed. The probe tip can penetrate beyond the most apical extent of the JE into the infiltrated connective tissue, when inflammation is present. After treatment and healing, with the deposition of new collagen, the probe tip will not reach the apical termination of the JE. From this

discussion it can be seen that an improvement in attachment level may not represent a true gain of attachment but only a resolution of inflammation (Nyman and Lindhe, 1997).

In spite of this drawback, measurement of attachment level is a useful tool for assessing response to treatment. In addition, AL is the most reliable method available of diagnosing the presence or absence of loss of periodontal support (Clerehugh and Lennon, 1984; Tonetti and Mombelli, 1997).

1.5.2.2 Radiographic analysis

The level and contour of the interdental alveolar bone can be assessed from examination of radiographs. The outline of the buccal and lingual alveolar bone may also be seen but will in many cases be masked by the teeth, restorations or by bone tissue (Nyman and Lindhe, 1997). For this reason and because radiographic evidence of loss or increase in bone density lags behind the clinical measurements, radiographic analysis must be combined with a detailed periodontal charting (Clerehugh and Lennon, 1986).

If the BL has progressed at a similar rate, the bone level will be even and is termed "horizontal bone loss". On the other hand, if BL has progressed at different rates around a particular tooth angular bone defects may be evident which are referred to as "vertical" bone loss. Alveolar bone loss is measured from the ACJ to a point where the lamina dura becomes continuous with the compact bone of the interdental septum. It is measured on the mesial and distal surfaces of the teeth. This is not a true measurement of BL since in health alveolar bone surrounds the tooth to a level approximately one millimetre apical to the ACJ. However, this is the most accurate method available of estimating the degree of BL.

1.5.3 Early Onset Periodontitis

The term early-onset periodontitis encompasses a group of diseases which are diagnosed in patients under the age of thirty-five years (Fourel, 1972; Astemborski *et al.*, 1989). The destruction of the periodontium is advanced for the age of onset of the

condition (Baer, 1971). Early-onset periodontitis has a tendency to aggregate in families (Hassell and Harris, 1995).

1.5.3.1 Prepubertal periodontitis

Page *et al.* (1983) first described this extremely rare category of periodontitis as a distinct clinical entity. The onset of the disease usually occurs during or soon after the eruption of the deciduous teeth. Both familial clustering of PP and a higher incidence in females have been documented (Fourel, 1972; Page *et al.*, 1983; Shapira, Schlesinger and Bimstein, 1997; Bimstein, Sela and Shapira, 1997).

Because of the rarity of this form of the disease, no strict definition of the level of AL and the number of teeth involved has been proposed. For this reason, no prevalence data exist for this condition. According to Page *et al.* (1983) the localised form affects some but not all of the primary teeth more often the molars. The associated plaque deposits are moderate and there is little inflammation of the gingivae, but bleeding on probing is present at affected sites. There are no associated systemic conditions and patients do not suffer from frequent upper respiratory tract infections. The destruction is not as rapid as in the generalised form and the condition usually responds to treatment.

Page *et al.* (1983) also described the following features of GPP. The generalised form affects all the deciduous teeth and sometimes also involves the permanent dentition. There is acute inflammation of the gingival tissues and rapid destruction of the periodontium often leading to premature exfoliation of the teeth. Pronounced recession and cleft formation are also sometimes present. The periodontitis may be refractory to therapy. Individuals may suffer from recurrent upper respiratory tract and skin infections. It has been suggested that this form of PP is only found associated with leucocyte adhesion deficiency syndrome (LAD) (Page *et al.*, 1983; Page, Beatty and Waldrop, 1987; Meyle, 1994). However, GPP has also been reported in otherwise healthy children (Butler, 1969; Fourel, 1972; Shapira, Schlesinger and Bimstein, 1997).

1.5.3.2 Localised early-onset periodontitis

This form of early-onset periodontitis is also referred to as localised juvenile periodontitis. There are numerous case definitions of LEOP adopted for convenience rather than being based on scientific evidence. The one presented here has been used in genetic studies of EOP. According to Hart *et al.* (1991) diagnosis of LEOP is based on AL of $\geq$ 4 mm on at least two permanent first molars and incisors (one of which must be a first permanent molar). Not more than two other permanent teeth, which are not first permanent molars or incisors, should be affected. Individuals must be systemically healthy and under 35 years of age.

1.5.3.3 Generalised early-onset periodontitis

The term GEOP covers various forms of the disease having overlapping clinical presentations, which can only be distinguished at present by observational longitudinal assessment. These include generalised juvenile periodontitis, generalised severe periodontitis and rapidly progressing periodontitis (RPP). Within each of these forms heterogeneity exists. The Early Onset Periodontitis workshop in 1996 recommended concentrating on immunological, bacteriological and environmental markers of disease (Van Dyke and Schenkein, 1996). In addition they recommended focusing on the earliest stage of detectable disease. The outcome of these proposals would help to correctly assign diagnoses, assess disease progression and elucidate the relationship between the localised and generalised forms of the disease.

GEOP has its onset from puberty until 35 years of age. According to Hart *et al.* (1991) clinical diagnosis is based on AL of $\geq$ 5 mm on a minimum of eight permanent teeth (one of which must be a first molar), at least three of which should not be first molars or incisors. Individuals must be systemically healthy.

1.5.3.4 Incidental early-onset periodontitis

Studies of prevalence of AL in adolescents and young adults have identified a substantial group of individuals who show a pattern of destruction which does not fit the classification of either LEOP or GEOP (Löe and Brown, 1991; Brown *et al.*, 1996;

Albandar *et al.*, 1997) (section 1.6.2). It is suggested that this category should include individuals who do not fulfil the criteria for diagnosis of either GEOP or LEOP, but show ≥ 4 mm of AL on one or more teeth. An epidemiological survey by Löe & Brown (1991) of early-onset periodontitis, in adolescents aged 14 to 17 years, was carried out in the United States. Because of the young age of the population sample AL of ≥ 3 mm was used as the cut-off for diagnosis of LEOP, GEOP and IEOP. It was found that 71% of the IEOP group had one site affected and 97% had three or less affected sites. This definition is a useful way of classifying those individuals who have a few sites affected by abnormal AL, which is not associated with local causes such as proximal caries or overhanging restorations.

1.5.4 Adult Periodontitis

Adult periodontitis is probably initiated at or soon after puberty but does not manifest symptoms until the middle of the fourth decade (Clerehugh, Lennon and Worthington, 1990). AP is a slowly progressing form of periodontitis. However, it may at any stage undergo an acute exacerbation with associated AL. From prevalence studies, AP is the most commonly occuring form of periodontitis. Varying levels of severity of AP exist. It is possible that AP patients with severe generalised disease, first diagnosed under the age of about 45 years, would have been classified as EOP had they been initially examined under the age of 35 years.

1.5.5 Refractory Periodontitis

The definition of refractory periodontitis includes all cases of EOP and AP which fail to respond to treatment and continue to lose attachment. It has been suggested that the majority of refractory periodontitis patients are smokers (MacFarlane *et al.*, 1992; Wolff, Dahlén and Aeppli, 1994). However, a recent study found this not to be the case (Colombo *et al.*, 1998a; 1998b).

1.5.6 Necrotising Ulcerative Periodontitis

This condition is preceeded by necrotising ulcerative gingivitis (NUG) which is an acute inflammatory condition associated with a fusospirochaetal microbiota. NUG is characterised by ulcerated and necrotic papillae and gingival margins (Holmstrup and Westergaard, 1997). The ulcers are covered by a grayish slough, removal of which causes bleeding from the exposed ulcers. The condition is painful and there is an associated foetor oris. As the necrosis progresses cratering appears between the buccal and lingual margins of the papillae. Untreated or recurrent NUG may progress to necrotising ulcerative periodontitis. This diagnosis follows the involvement of the periodontal ligament and the destruction of the interproximal alveolar bone (Holmstrup and Westergaard, 1997). Marked gingival recession may result and sequestra formation is a sequela of the rapid necrosis. In industrialised countries young adults in their early 20s appear to be most predisposed to this condition and a prevalence of 0.5% or less has been reported (Horning, Hatch and Lutskus, 1990).

1.5.7 Periodontitis Associated with Systemic Disease

Severe periodontitis of the deciduous and permanent dentitions has been associated with the monogenic disorders and also with Down's syndrome (section 1.8.3.1, 1.8.4.1 and 1.8.5.1). In addition, patients with both insulin-dependent-diabetes mellitus and non-insulin dependent diabetes mellitus have been shown to be at greater risk of developing periodontitis (Genco and Löe, 1993; Grossi *et al.*, 1994; Oliver and Tervonen, 1994; Page and Beck, 1997).

Another systemic condition, which demonstrates an increased risk of developing periodontitis, is infection with human immunodeficiency virus (HIV). Necrotising ulcerative periodontitis has also been associated with the late stages of this condition (Holmstrup and Westergaard, 1994).

1.6 Prevalence of Loss of Periodontal Support in Individuals less than Thirty-five Years of Age

1.6.1 Introduction

The following section presents a review of prevalence of loss of periodontal support in children, adolescents and young adults. It attempts to illustrate the rarity of severe generalised disease in individuals under the age of 35 years. It also demonstrates that mild AL ($\geq$ 1 mm and < 3 mm) and marginal BL ($\geq$ 2 mm) of the permanent dentition are relatively common in circumpubertal children and teenagers (Hansen, Gjermo and Bergwitz Larsen, 1984; Clerehugh, Lennon and Worthington, 1990; Neely, 1992).

Case definitions of localised, generalised and incidental EOP have been presented in the previous section on classification of periodontal disease. However, epidemiological studies in children and young adults suffer from a lack of accepted definitions of the various forms of the disease. Wide variation in prevalence of loss of periodontal support exists between studies, due to the use of different diagnostic criteria and overlap between the disease categories. This has confounded meaningful comparison of epidemiological reports.

1.6.2 Longitudinal Studies

Several longitudinal studies have highlighted the dynamic nature of periodontal AL (Albandar, Buishi and Barbosa, 1991; Albandar, 1993; Albandar *et al.*, 1997; Gunsolley *et al.*, 1995; Brown *et al.*, 1996). It appears that some cases progress with time to involve more teeth and the deeper structures of the periodontium, whereas in other cases the disease arrests or healing occurs. These observations highlight the marked heterogeneity of the disease.

It has been proposed that the most sensitive method of diagnosis of periodontal disease is the measurement of AL by probing (Tonetti and Mombelli, 1997). A five year longitudinal study was carried out on 167 adolescents of a low socio-economic group who were considered likely to develop periodontitis (Clerehugh, Lennon and Worthington, 1990). The children were fourteen years of age at the start of the study.

AL was measured on the mesio-buccal surfaces of first molars, first premolars and central incisors. The prevalence of AL of $\geq$ 1 mm was calculated at 14.3 years, 16.0 years and 19.6 years. The subject prevalence was found to be 3%, 37% and 77% and the site prevalence < 1%, 7% and 31%, respectively. It has been shown that AL of $\geq$ 1 mm correlates with early radiographic BL (Clerehugh and Lennon, 1986). AL of $\geq$ 2 mm $\leq$ 3 mm was only found at 19 years and affected 14% of the population. Individuals who had evidence of AL by 16 years still had significantly more sites affected by the age of 19.6 years. It appears therefore that AL of $\geq$ 1 mm is widespread in adolescents. In addition, in a significant proportion of these individuals AL progresses to $\geq$ 2 mm by the late teens. This group may be indicative of prevalence of slowly progressing AP diagnosed during the fourth decade, since AL of 1-3 mm is a valid measure of early periodontitis (Clerehugh and Lennon, 1984; Clerehugh, Lennon and Worthington, 1988; Albandar, 1993).

Two recent papers investigated clinical classification of periodontitis in a sample of adolescents and young adults from the 1986/1987 survey of 14,013 North American schoolchildren (Brown *et al.*, 1996; Albandar *et al.*, 1997). The subjects were examined at baseline, when aged between 13 and 20 years old, and at follow-up six years later to determine whether diagnoses, based on cross-sectional assessment, change over time. In the classification of Löe and Brown (Löe and Brown, 1991; Brown *et al.*, 1996; Albandar *et al.*, 1997) AL of $\geq$ 3 mm on the mesial surface of any tooth, excluding third molars, was taken as evidence of significant disease. Individuals were classified into three groups. Subjects required at least one first molar and at least one incisor or second molar, and two or fewer other teeth, to be placed in the LEOP group. For GEOP (if the LEOP criteria were not met) the selection criteria were four or more teeth with AL $\geq$ 3 mm, and a minimum of two affected teeth which were second molars, canines or premolars. Individuals who did not meet the criteria for either LEOP or GEOP, but showed AL of $\geq$ 3 mm on one or more teeth, were included in the IEOP group. The results indicated that 82% of cases of GEOP remained as such when re-classified at follow-up and 35% of cases of LEOP progressed to GEOP. Twenty-eight percent of IEOP subjects progressed to either LEOP or GEOP and 30% either did not progress or healed. It can be seen from these data that progression of EOP does not occur in a straightforward linear fashion.

1.6.3 Cross-Sectional Studies

In a recent large-scale study of periodontal status in the United States, prevalence rates for a minimum of one site with clinical AL of ≥ 1 mm, ≥ 3 mm and ≥ 5 mm were recorded (Brown, Brunelle and Kingman, 1996). A total of 7,447 individuals were examined, representing 160.3 million civilian, non-institutionalised Americans. Only 0.3% of individuals in the 13-17 and 18-24 year age groups were found to be affected by AL of ≥ 5 mm. In the 25-34 year age group the number affected was 6.4% of the population. This figure appears to be unusually high. However, it should be borne in mind that in an earlier study, almost two-thirds of those individuals diagnosed as being affected demonstrated incidental AL (Löe and Brown, 1991). The bias towards this form of AL is probably also reflected in the 25-34 year age group.

Another recent cross-sectional survey in North America, of 5,849 individuals aged 18-34 years found 3.6% had AL of ≥ 3 mm on at least eight teeth (Oliver, Brown and Löe, 1998). The authors classified this level of disease as moderate GEOP. For subjects aged between 25 and 35 years these criteria appear to lack precision and may overlap with AP. A prevalence of only 0.3% was found for advanced GEOP where AL of ≥ 5 mm was present on eight teeth. However, these inclusion criteria may miss potentially severe disease at the younger end of the age range, since an increase in frequency of both moderate and severe GEOP was found with age. It is also worth mentioning that the validity of these American studies has been questioned. Only the mesio-buccal and mid-buccal sites on all fully erupted permanent teeth in two randomly selected quadrants (one maxillary and one mandibular) were examined in each individual, which may underestimate severe disease.

Assessment of AL by probing may be difficult in the mixed dentition. Measurement from the amelocemental junction to the alveolar crest on bite-wing radiographs has therefore been suggested as a screening method in prepubertal children (Tonetti and Mombelli, 1997). Needleman *et al.* (1997) found median distances at primary molars were 0.58-1.39 mm in seven- to nine-year-old children. In another study ACJ to alveolar crest measurements were symmetrically distributed around the mean of 0.9 mm with a range of 0.0 - 3.0 mm (Sjödin and Matsson, 1992). It was concluded that

sites with ACJ to alveolar crest measurements of more than 2.0 mm showed pathological BL. Sjödin and Matsson (1994) conducted an epidemiological survey of radiographic bone levels of 2017 Swedish children of nine years of age. They found that 4.5% showed marginal BL of ≥ 2.0 mm from the amelocemental junction to the alveolar crest, affecting deciduous molars and canines. Another study carried out by the same group compared marginal BL ≥ 2.0 mm in the primary dentition with the same amount of BL in the permanent dentition, in individuals aged 13 to 19 years (Sjödin *et al.*, 1993). They found that 20% of patients with one site with BL in the permanent dentition had evidence of BL in the primary dentition, between the age of five and 12 years. The figure for individuals with more than two sites was 52%. A healthy reference group had 5% of their subjects who showed BL in the deciduous dentition. These data indicate that abnormal BL in adolescents and young adults may be associated with BL in prepubertal children.

A study in North America of circumpubertal children aged 10 to 12 years screened bite-wing radiographs for evidence of interproximal BL of ≥ 2 mm. Ten percent of children were selected, but a prevalence rate of only 0.45% was found for AL of ≥ 3 mm on at least one first permanent molar, following clinical examination (Neely, 1992).

Saxén (1980c) found a prevalence of 0.1% for loss of periodontal support in a population of 8,096, 16-year-old, Finnish schoolchildren. Screening was initially performed using bite-wing radiographs. Individuals who displayed interdental BL of ≥ 2 mm around more than one tooth were selected for a detailed clinical examination. Out of 28 cases selected, diagnosis was confirmed in eight individuals. Probing pocket depth measurements of ≥ 3 mm and subsequently radiographic re-examination, six months to two years after the initial examination, ratified the diagnosis. The ethnic group studied was said to represent a uniform Caucasoid population. It has been suggested that bite-wing radiographs are a useful method of detecting early bone lesions in adolescents (Blankenstein, Murray and Lind, 1978; Hansen, Gjermo and Bergwitz Larsen, 1984). However the level at which a threshold should be set to determine only those individuals who will develop severe disease is uncertain. Evidence of loss of periodontal support from radiographic examination requires to be corroborated by clinical examination.

1.6.4 Racial Variation in Loss of Clinical Attachment

Loss of periodontal support in adolescents and young adults occurs at least fifteen times more frequently in blacks than in Caucasians (Saxby, 1987; Astemborski *et al.*, 1989; Löe and Brown, 1991; Melvin, Sandifer and Gray, 1991). It is also more common in Hispanics than non-Hispanics (Ismail *et al.*, 1987; Löe and Brown, 1991). Saxby (1984b; 1987) used the same diagnostic criteria as Saxen to assess loss of periodontal support. He found an overall prevalence of 0.1% in a mixed ethnic population of 7,266, 15 to 19 year-olds, from the west Midlands. When the population was sub-divided into its separate ethnic groups, prevalence of 0.02% was shown for Caucasians, 0.8% for Afro-Caribbeans and 0.2% for Asians.

Few epidemiological studies of adolescents exist where localised is distinguished from generalised AL. This may be because of the very low occurrence of generalised AL in this age group. Löe and Brown (1991) conducted a large-scale survey of 11,000 North American schoolchildren aged between 14 and 17 years. The diagnostic criteria have been previously described in section 1.6.2. They found a prevalence of 0.53% for LEOP, 0.13% for GEOP and 1.16% for IEOP. When the dataset was split into defined racial groups, prevalence of 2.05% for black and 0.14% for whites was found for LEOP. A prevalence of 0.59% and 0.03% respectively was found for GEOP. Incidental EOP was also five times more frequent in blacks than whites. In addition, Hispanics were 2.4 times more likely to have LEOP than non-Hispanics.

1.6.5 Sex Predilection

A number of studies have found prevalence of loss of periodontal support to be evenly distributed between the sexes (Saxén, 1980a; Saxby, 1984a; Melvin, Sandifer and Gray, 1991). However some investigators have found variations in prevalence between males and females in different racial groups. In a population of adolescents in Amsterdam a prevalence of 0.35%, with loss of attachment of $\geq$ 5mm on at least one tooth, was demonstrated (van der Velden *et al.*, 1989). This study showed a predilection for female subjects of 1.3:1.0, but no racial differences. This may have been because 82% of the population were white Caucasians.

Black males have been found 2 to 3 times more likely to have localised AL than black females (Harley and Floyd, 1988; Löe and Brown, 1991; Melvin, Sandifer and Gray, 1991). Caucasian females have an odds ratio (OR) 1 to 2.5 times that of Caucasian males (Saxén, 1980a; 1980c; van der Velden *et al.*, 1989; Löe and Brown, 1991). For generalised loss of attachment, males manifest the disease more often than females by a ratio of 4.3:1 (Löe and Brown, 1991).

1.6.6 Summary

A review of the literature, on prevalence of loss of periodontal support in individuals under the age of 35 years, uncovers the confusion that exists regarding diagnostic criteria for the various forms of EOP. Bearing this point in mind, it appears that the prevalence of severe generalised AL is rare in this age group. The severity and extent of AL increases with age. Both localised and generalised loss of periodontal support is found between 15 and 40 times more commonly in blacks than Caucasians. Sex predilection varies between racial groups. Furthermore, a large proportion of teenagers show mild AL of $\geq$ 1mm and a significant proportion show AL of $\geq$ 2 mm but $\leq$ 3mm by their late teens. The latter group probably does not represent those who have a predisposition to develop severe AL under the age of 35 years, but may represent the initiation of slowly progressing AP. Another interesting finding is the high prevalence of incidental AL, compared with the localised and generalised forms, in younger age groups.

1.7 Smoking and Periodontal Disease

1.7.1 Introduction

Dentists believed for decades that those patients who smoked showed a poorer standard of oral hygiene and accumulated more calculus on their teeth than non-smokers. Smoking was also implicated as a risk factor for acute necrotising ulcerative gingivitis (Pindborg, 1947). There were however, until more recently, few studies of the relationship between smoking and chronic inflammatory periodontal disease. In

the past 15 years, compelling evidence has revealed a strong association between smoking and periodontitis. Even after adjustment for factors such as oral hygiene status, age, gender, education and socio-economic status, a greater risk for periodontitis exists among smokers. Epidemiological studies in a number of different countries across the world have corroborated these findings.

1.7.2 Cross Sectional Studies

Numerous cross-sectional epidemiological studies of the relationship between smoking status and periodontal health have been reported. A large-scale survey in North America of 1,426 subjects aged between 25 and 74 years assessed risk for AL and BL (Grossi *et al.*, 1994; 1995). Smoking had relative risks for AL ranging from 2.05 (95% confidence interval [CI]: 1.47-3.24) in light smokers up to 4.75 (95% CI: 3.28-6.91) in heavy smokers (Grossi *et al.*, 1994). For severe BL the range was from 3.25 (95% CI: 2.33-4.54) to 7.28 (95% CI: 5.09-10.31) in light to heavy smokers (Grossi *et al.*, 1995).

Two studies in Sweden by Bergström and co-workers (Bergström and Eliasson, 1987; Bergström, Eliasson and Preber, 1991) investigated smoking and BL in individuals with a high standard of oral hygiene. The first study of 235 professional musicians, 21 to 60 years of age, found alveolar bone height was significantly lower in smokers (n = 72) compared with non-smokers (n = 163). Former smokers were excluded from the study. When age and oral hygiene were taken into consideration the findings were still unchanged (Bergström and Eliasson, 1987). The second study was of dental hygienists and included 63 current smokers, 67 former smokers and 80 non-smokers, aged 24 to 60 years (Bergström, Eliasson and Preber, 1991). Interproximal bone height was determined on bite-wing radiographs. BL was significantly greater for smokers than non-smokers. There was no significant difference in bone height between former smokers and non-smokers. Oral hygiene status including the presence or absence of calculus showed no relationship to smoking. The authors concluded that oral hygiene does not vary with smoking when study and control groups are matched for socio-economic status.

Linden and Mullally (1994) investigated the association between smoking and periodontal disease in 82 regular dental attenders (21 current smokers and 61 non-smokers) aged between 20 and 33 years. They found that smokers had similar levels of plaque to non-smokers but had more interproximal subgingival calculus. In addition, smokers had significantly more pocketing of ≥ 4 mm and AL of ≥ 2 mm than non-smokers. Severe AL of ≥ 6 mm was recorded in 4 (19%) smokers and 2 (3%) non-smokers.

The effect of smoking on periodontal health was assessed in diabetic and non-diabetic individuals aged between 19 and 40 years (Haber *et al.*, 1993). In the non-diabetics, periodontitis was observed significantly more often among current smokers compared with non-smokers. Similar effects were observed in the diabetic group. Furthermore, half of the periodontitis in non-diabetics aged between 19 and 30 years and one third in 31 to 40 year olds was estimated to be due to smoking. No differences were observed between smokers and non-smokers with regard to oral hygiene status.

Another interesting study by Haber and Kent (1992) compared the prevalence of smoking between patients from a periodontal practice (mean age = 51.5 ± 11.9 years) and general dental practice patients (mean age = 43.5 ± 13.6 years). Only patients who were periodontally healthy were recruited from general practice. The percentage of combined former and current smokers with moderate to severe periodontitis (75%) was higher than the percentage reported by patients from general practice (54%) across all age groups. The age- and sex-adjusted OR for having a positive smoking history among the moderate to severe periodontitis patients versus the general practice subjects was 2.6. Furthermore, a significantly higher number of periodontal patients were heavy smokers ($\geq$ 10 cigarettes per day) than general practice patients (OR = 5.7).

A case-control study of the relationship between life-events and periodontitis found smoking to be associated with periodontitis ($p < 0.01$) (Croucher *et al.*, 1997). However after controlling for oral hygiene status and socio-demographic variables smoking was no longer significantly associated with periodontal disease. In the discussion the authors suggested that this may have been due to smoking being a

confounding factor for several other variables in the model and overadjustment having occurred. Another recent study examined psychosocial factors, dental plaque levels and smoking in AP and RPP patients (Monteiro da Silva *et al.*, 1998). It was shown that no differences in oral hygiene status existed between the two groups. However, RPP patients smoked significantly more tobacco than AP patients ($p = 0.02$). These findings confirm earlier studies demonstrating a higher risk of disease in younger adult smokers (Haber *et al.*, 1993; Linden and Mullally, 1994).

A recent large-scale epidemiological survey of the dental status and smoking habits of randomised samples of 35, 50, 65 and 75 year-old subjects was undertaken in Sweden (Axelsson, Paulander and Lindhe, 1998). The results showed that smokers had a higher mean AL than non-smokers across all ages. The difference between the two groups in mean AL increased from 0.37 in 35 year-old subjects to 1.33 in 75 year olds. This finding confirmed the results of other studies, which showed a cumulative effect of smoking (Bergström and Eliasson, 1987; Bergström, Eliasson and Preber, 1991; Martinez-Canut, Lorca and Magán, 1995).

It has been observed that approximately 90% of patients who are refractory to periodontal therapy are smokers (MacFarlane *et al.*, 1992; Wolff, Dahlén and Aeppli, 1994). Two recent studies by Colombo and co-workers (Colombo *et al.*, 1998a; 1998b) found that only a quarter of their population of refractory patients were smokers. However, around 40% of this population were former smokers. Another group suggested that former smokers often relapse and therefore self-reporting is not an accurate measure of smoking exposure (Boström, Linder and Bergström, 1998b) (section 1.7.3.3).

Taken together the findings of these studies suggest a strong association between smoking and periodontal health, regardless of oral hygiene status. It appears the misconception that arose, in which smokers exhibited poorer levels of oral hygiene, may have been due to a lack of consideration of confounding variables such as socio-economic status. Cross-sectional studies of associations between possible risk variables and any disease can indicate factors which may increase the likelihood of a

disease occurring (Page and Beck, 1997). In order to confirm a particular characteristic as a risk factor, observational longitudinal studies need to be undertaken.

1.7.3 Longitudinal Studies

1.7.3.1 Observational studies

A report of AL over five years in community-dwelling older adults found smokers to be at increased risk of AL (Beck *et al.*, 1997). In another study of prognostic factors in periodontal disease over a one-year period, smokers were found to show greater AL and BL than non-smokers. Smoking was suggested to significantly increase the odds of showing further AL by 5.4:1.0 (Machtei *et al.*, 1997).

1.7.3.2 Response to treatment

Studies of response to treatment have demonstrated similar findings. Clinical and microbiological effects of non-surgical therapy were investigated in smokers and non-smokers (Preber, Linder and Bergström, 1995). The authors found that smokers showed a less favourable outcome in probing depth reduction despite a similar decrease in the presence of periodontal pathogens in the two groups. Another study investigated changes in alveolar crestal height 11 to 14 months following the completion of hygiene phase therapy (Machtei *et al.*, 1998). Non-smokers showed no change in bone height but smokers continued to lose bone at an annual rate of 0.17 mm ($p < 0.005$).

Reports of various surgical procedures including conventional surgery (Preber and Bergström, 1990), guided tissue regeneration procedures (Tonetti, Pini-Prato and Cortellini, 1995; Trombelli and Scabbia, 1997) and implant placement (Haas *et al.*, 1996), have also showed a less favourable clinical response in smokers compared with non-smokers.

In a recent study of a 5-year maintenance programme following surgery, smokers were found to exhibit less improvement than non-smokers in terms of bone height. Smokers were also more likely to show BL after five years of maintenance than non-smokers

(Boström, Linder and Bergström, 1998a). An earlier report had demonstrated that smokers show an increased likelihood of recurrence of disease following therapy than non-smokers (Kaldahl *et al.*, 1996).

1.7.3.3 Smoking Cessation

An improvement in periodontal health following smoking cessation provides further evidence for smoking as a risk factor in periodontitis. A study designed to assess the prevalence of cigarette smoking in a group of moderate to severe AP patients from a periodontal practice and a group of periodontally healthy patients from general dental practice illustrated this point (Haber and Kent, 1992). Smokers were split into former smokers and current smokers. The OR between the periodontal practice patients and the general practice subjects, for non-smokers versus former smokers and non-smokers versus current smokers, were 2.1 and 3.3 respectively. A longitudinal report of BL over ten years showed that progression of destruction was significantly slowed in subjects who gave up smoking during the study, compared with those who continued to smoke (Bolin *et al.*, 1993).

Several studies have demonstrated more missing teeth in smokers than non-smokers (Bergström and Floderus-Myrhed, 1983; Linden and Mullally, 1994; Holm, 1994; Axelsson, Paulander and Lindhe, 1998). Krall *et al.* (1997) confirmed this finding and reported that current smokers had more missing teeth than either former smokers or never smokers. Prospective observations over six years of 1231 male veterans, aged between 21 and 75 years, found that the rate of tooth loss was significantly reduced in individuals who stopped smoking during the study. However, the rate remained higher than in non-smokers (Krall *et al.*, 1997).

To summarise, the findings of longitudinal epidemiological surveys, studies of response to treatment and reports of smoking cessation, provide further convincing evidence of a strong association between smoking and periodontal health. The confirmation of smoking as a risk factor for periodontitis awaits the publication of further prospective longitudinal studies of the effects of smoking cessation on clinical periodontal parameters. In future studies of smoking cessation, cigarette exposure should be assessed by assaying serum cotinine levels. Cotinine is a metabolite of

nicotine. This approach would improve the accuracy of smoking studies, since patient compliance and self-reported smoking status are unreliable (Gonzalez *et al.*, 1996).

1.7.4 Dose Response

The relationship between smoking and periodontal disease appears to be a dose response effect and is also cumulative. Most studies have measured smoking exposure in pack years i.e. the number of packs of cigarettes smoked per day x the number of years smoked. In calculating this figure it should be remembered that current smoking levels may not reflect past exposure. However, in spite of this drawback reports have consistently demonstrated a dose response for smoking in relation to periodontitis.

Grossi and co-workers (Grossi *et al.*, 1994; 1995) found that both AL and BL increased with increasing pack years. They found that the healthy, low, moderate, high and severe AL groups showed increasing number of mean pack years from 4.3 in the healthy group to 28.3 in the severe group (Grossi *et al.*, 1994). The range was 5.3 to 26.8 mean pack years from the healthy to severe BL groups (Grossi *et al.*, 1995). Another study in Sweden of dental hygienists showed most of the difference in BL between smokers and non-smokers appeared to be due to those who smoked more than 10 pack years (Bergström, Eliasson and Preber, 1991). In addition, in the younger age group (< 30 years) there was no difference in bone levels between smokers and non-smokers.

In a large scale survey of 899 Spanish periodontal patients (age range 21 to 76), of whom 47.4% were non-smokers and 52.6% were smokers, the influence of smoking on disease severity was assessed (Martinez-Canut, Lorca and Magán, 1995). There was no significant difference in AL between smokers of less than 10 cigarettes per day (AL = 3.72 mm $\pm$ 0.86) and non-smokers (AL = 3.84 $\pm$ 0.89). However, smokers who smoked more than 11 but less than 20 (AL = 4.36 $\pm$ 1.23) and more than 20 cigarettes per day (AL = 4.50 $\pm$ 1.04) showed significantly more AL than non-smokers.

Another large-scale study investigating the association between cigarette smoking and infection with specific periodontal pathogens was carried out in North America (Zambon *et al.*, 1996a). Of the 1,426 subjects who took part, 798 were current or

former smokers. Smokers were found to be infected by significantly higher levels of Bf and were at significantly higher risk of subgingival infection by this organism than non-smokers. The risk in current smokers was 2.3 times that of former or non-smokers. In addition, the relative risk of infection by Bf increased 1.18 times for every category of smoking from very light to heavy. The authors suggested that this increased risk of subgingival infection with periodontal pathogens might explain in part, the increased risk for periodontal disease seen in smokers.

1.7.5 Clinical Effects

Smoking has pronounced effects on the gingival tissues in periodontitis, by suppressing the clinical signs of gingivitis. The gingivae of smokers instead of appearing inflamed and swollen may be pale, fibrotic and hyperkeratotic, compared with non-smokers exhibiting equal levels of disease. In addition, the gingival papillae are blunted and thickened with rolled margins, which is a characteristic feature of smokers with or without periodontitis. Smokers have a reduced tendency to bleed on gentle probing into the periodontal pocket and produce significantly less GCF (Bergström and Floderus-Myrhed, 1983; Holmes, 1990). It has been suggested that nicotine causes vasoconstriction of the peripheral blood vessels in the gingivae (Clarke, Shephard and Hirsch, 1981). Smokers also show more advanced disease anteriorly especially on the palatal surfaces of the incisors and canines. Destruction tends to be more severe and widespread in smokers compared with non-smokers of the same age. Finally, smokers have been shown to demonstrate more recession than non-smokers, whether they have periodontitis or not (Martinez-Canut, Lorca and Magán, 1995; Gunsolley *et al.*, 1998). However, it has been suggested that this finding could be due to toothbrush abrasion rather than the effects of smoking. Smokers exhibit more staining than non-smokers and may use harder toothbrushes to clean their teeth (Gunsolley *et al.*, 1998).

As already mentioned in a previous section, evidence from studies of response to treatment suggests that smokers respond less well to therapy than non-smokers. One possible explanation for this effect may be the result of the resolution of gingival swelling in non-smokers, causing a more pronounced reduction in probing depth

compared with smokers. However, this scenario would only apply to longitudinal studies of initial hygiene phase therapy. In reports of response to surgical treatment, shrinkage and recession, due to healing of the marginal gingivae, would have occurred prior to the start of the study. A recent paper by Biddle *et al.* (1998) raised the possibility that the smaller improvement in attachment level and probing pocket depth, seen in smokers, may be due to less probe tip penetration at baseline. The reduced inflammation and supra-bony connective tissue at the base of the pocket in smokers may partly explain this finding. This interpretation would apply to studies of response to treatment of both non-surgical and surgical therapy.

1.7.6 The Effect of Smoking on the Host Response in Periodontitis

Numerous aspects of the host response may be modified by the effects of smoking, tobacco and nicotine. A recent review by Barbour *et al.* (1997) discusses in detail the immune and inflammatory mechanisms affected by smoking and tobacco products, which may possibly play a role in periodontal disease. The following paragraphs will concentrate on those features of host defence for which there is some evidence of an association between smoking and periodontitis.

1.7.6.1 Effects on specific cell types

In vitro experiments have shown that nicotine can affect the proliferation and adherence of human gingival fibroblasts (Peacock *et al.*, 1993). Chemotactic responses of gingival neutrophils may be enhanced or suppressed when exposed to nicotine (Kraal *et al.*, 1977; Totti *et al.*, 1984). However phagocytic function appears to be reduced (Kenney *et al.*, 1977). This finding was corroborated more recently in a study of patients with refractory periodontitis, of whom 90% were smokers. In this report neutrophil adherence to and phagocytosis of periodontal pathogens was suppressed (MacFarlane *et al.*, 1992; Wolff, Dahlén and Aeppli, 1994).

Smoking may also affect the recruitment of inflammatory and immune cells to the gingival tissues by altering expression of adhesion molecules. Smoking has been found to increase the amount of soluble ICAM-1 in the serum (Koundouros *et al.*, 1996).

1.7.6.2 Inflammatory mediators

It has recently been shown that TNFα levels in the GCF of periodontal maintenance patients, five years after periodontal surgery, were significantly higher in smokers than non-smokers (Boström, Linder and Bergström, 1998a). A further study by this group assessed the same parameters in patients with untreated moderate to advanced periodontitis (Boström, Linder and Bergström, 1998b). It was demonstrated that in this disease category also, smoking was significantly associated with increased TNFα level in GCF ($p = 0.001$). In addition, 90% of current smokers, 68% of former smokers and 51% of non-smokers showed detectable levels of TNFα. The authors suggested that the increased TNFα levels exhibited by smokers may be indicative of more destructive disease amongst smokers. They also proposed that the increased levels of the cytokine among former smokers may have been due to periodical relapse to smoking in this group. This observation gives further credence to the use of the serum cotinine assay for accurately determining smoking exposure.

Another recent study examined the effect of nicotine and smokeless tobacco (ST) on GMC and PBMC from non-smoking AP patients (Bernzweig *et al.*, 1998). PGE_2 secretion was found to be increased in PBMC after stimulation with nicotine and ST, but no effect on IL-1β was observed. Secretion of PGE_2 from GMC could not be increased further when stimulated with nicotine, ST or LPS. This indicated that GMC were already maximally upregulated by the inflammation present in the periodontal lesion. Levels of IL-1β from GMC were decreased by exposure to nicotine compared with controls. The authors suggested a potential regulatory role for nicotine in periodontal disease of smokers (Bernzweig *et al.*, 1998). However, the levels of nicotine used in these experiments (100μg/ml) were well above the levels found in serum in cigarette smokers (72 ng/ml). Furthermore, even though the nicotine levels in saliva of smokers have been found to be higher (1.3 μg/ml) than in serum, they did not approach the levels used in this study. These findings should therefore be interpreted with caution.

1.7.6.3 The humoral immune response

Antibodies of the IgG2 isotype are thought to play an important role, in the protective humoral immune response in periodontitis. They are directed against carbohydrate antigens found on the surfaces of periodontal pathogens. IgG2 levels have been found to be significantly reduced in smokers with AP, GEOP and in those with a healthy periodontium, compared to non-smokers (Tew *et al.*, 1996). More specifically, IgG2 titres directed against Aa in black smokers are suppressed in GEOP patients but not in those with LEOP (Tangada *et al.*, 1997). Furthermore, the lower IgG2 titres seen in black GEOP smokers may be specific to Aa. IgG2 response against antigens of other non-periodontal bacteria may not be lower in GEOP subjects who smoke.

Gunsolley *et al.* (1997) recently investigated the effects of race, smoking and immunoglobulin allotypes on IgG subclass concentration in various diagnostic categories of periodontitis and in healthy controls. In black AP patients IgG1 was lower in smokers, while those with GEOP showed lower IgG2 levels, confirming earlier reports. In white subjects, complex relationships between smoking and allotypic markers were reported but none of these effects influenced periodontal diagnosis. However subsequently the same research group found white AP patients and healthy controls who smoked had higher mean AL and lower IgG2 levels than non-smokers (Quinn *et al.*, 1998). Conversely there were no differences in these parameters between black AP smokers and their controls.

Recently, subgingival infection with Bf has been shown to occur significantly more frequently in smokers (Zambon *et al.*, 1996a). In addition, smoking and subgingival infection with *Prevotella intermedia* (Pi), Bf and Pg were found to be significant prognostic indicators for future periodontal destruction (Machtei *et al.*, 1997). Two earlier studies had found no significant differences in prevalence of Pg, Aa, Pi, *Eikenella corrodens* (Ec) or Fn between smokers and non-smokers (Preber, Bergström and Linder, 1992; Stoltenberg *et al.*, 1993). Further investigations into the association of specific periodontal pathogens with smoking should help to elucidate these findings.

1.7.7 Summary

Many well-designed epidemiological, cross-sectional studies carried out in the last 15 years, have provided convincing evidence of an aetiological role for smoking in periodontal disease. Smokers appear to be in the region of 2.5 to 6.0 times more likely to develop periodontitis than non-smokers (Barbour *et al.*, 1997). More recently, longitudinal observational studies and reports of response to treatment have supported the findings of cross-sectional data. Studies of smoking cessation also add to this body of evidence.

A significant dose response effect of smoking has been observed. A number of reports have shown that light smokers (< 5 pack years) have no increased risk of periodontal disease than non-smokers. However, more heavy smokers have periodontitis than a healthy periodontium.

The manner in which smoking affects the host defence system and increases susceptibility to periodontitis is unclear. Suppression of inflammatory and resident cells, stimulation of proinflammatory mediators and down-regulation of the humoral immune response have been proposed as possible mechanisms. In addition smoking, race and disease category appear to affect IgG2 response in periodontal patients. Therefore, both environment and genotype influence the severity of disease observed in patients with periodontal disease. Research in this area in the future will help to unravel the complex processes involved.

1.8 Genetic Predisposition to Early Onset Periodontitis

1.8.1 Introduction

This review of genetic predisposition to early-onset periodontitis is divided into five sections. The first section presents an overview of study approaches used to investigate the role of genetic factors in periodontal disease. The three following sections summarise the current status of research into the genetic basis of prepubertal periodontitis, localised and generalised early-onset periodontitis, and adult

periodontitis. The final section brings together reports of genetically determined host response aspects which have been investigated in periodontitis patients.

1.8.2 Overview of Study Approaches

Evidence for a genetic predisposition to periodontitis comes from four areas of research: 1) the study of inherited diseases and genetic syndromes; 2) association studies; 3) twin studies; and 4) family studies. In this section the design, intent and interpretation of each of these four approaches to the study of genetic diseases will be discussed.

1.8.2.1 Study of inherited diseases and genetic syndromes

Evidence for the role of specific genes in disease may be gleaned from the study of inherited conditions or genetic disorders, in which the disease is pathognomonic. A number of monogenic syndromes with accompanying severe periodontal disease have been reported in the literature. Although these reports have not always distinguished disease categories, they have identified the striking and consistent co-occurrence of early-onset and severe periodontitis. Examining the underlying immunological or structural defects associated with the inherited conditions and genetic syndromes can provide insight into the pathogenesis of disease in the general population.

1.8.2.2 Association studies

Environmental or behavioural risk factors for a disease are often first detected in large epidemiological or population-based studies. In genetic epidemiology, similar approaches can be used to identify genetic risk factors for disease. The frequencies of polymorphisms of candidate genes, whose protein products play a role in the inflammatory or immune response, can be compared between unrelated cases and controls. A significant difference in the frequency of a specific polymorphism between a disease group and a control group is evidence that the candidate gene plays some role in determining susceptibility to disease. An association indicates that either the candidate gene directly affects disease susceptibility or that it is in linkage disequilibrium with (very close to) the disease locus. This method can help to

elucidate the pathogenesis of a disease process, identify aetiological heterogeneity and ultimately identify individuals most at risk of disease (Sofaer, 1990). In population studies it is important to clearly define disease status. Likewise, because of the possibility of racial heterogeneity (Marazita *et al.*, 1994), it is important to insure that patient and control groups are racially matched.

1.8.2.3 Twin studies

Studying phenotypic characteristics of twins is a method of differentiating variations due to environmental and genetic factors. Sir Francis Galton in 1875 was the first scientist to use this concept to study disease aetiology. The twin method has been applied to many spheres of medical research including Alzheimer's disease (Räihä *et al.*, 1996), cancer (Ahlbom *et al.*, 1997), diabetes (Poulsen *et al.*, 1997) and response to drug administration (Vojvoda *et al.*, 1996). Monozygous (MZ) twins arise from a single fertilised ovum and are therefore genetically identical and always the same sex. Dizygous (DZ) twins arise from the fertilisation of two separate ova and share, on average, one half of their genes in the same way as siblings do. Any discordance in disease between MZ twins must be due to environmental factors. Any discordance between DZ twins could arise from environmental and/or genetic variance. Therefore, the difference in discordance between MZ and DZ twins is a measure of the effects of the excess shared genes in MZ twins, when the environmental influence is constant.

In studying twins reared-together it is assumed, and can be indirectly tested, that the environmental variances of MZ and DZ twins are similar. For a condition which is predominantly environmental in origin, the correlations of MZ and DZ twins would be expected to be similar. If host genes influence disease susceptibility, concordance rates would be greater in MZ than DZ twins.

Studying MZ twins separated at birth and reared-apart provides an alternative approach to determining the concordance due to shared genes (Bouchard, 1984). Under the assumption that adoption is independent of related environmental factors, then the similarity of these twins is due solely to the effects of shared genotype. The rarity of such twins has limited their use in medical studies.

1.8.2.4 Family studies

Many diseases and disorders aggregate in families. The magnitude of clustering, or family risk, can be estimated by comparing the risk of disease in first degree relatives of patients to the risk of disease in the general population (Vyse and Todd, 1996). A risk ratio of 1.0 implies no familial clustering. Since family members share descendent genes and environments, data from extended families can be used to separate the relative influences of genetic and environmental factors on the disease or disorder.

Most of the evidence to date for a genetic predisposition to EOP comes from segregation analysis. This method is used to search for a major gene. It attempts to uncover a pattern in family data that corresponds to the transmission of a gene (or in rare cases multiple genes). The observed proportions of affected siblings and offspring are compared with expected proportions under specific genetic hypotheses. For example, an X-linked recessive hypothesis would predict that males would be predominantly affected. The terms pedigree analysis and segregation analysis are often used interchangeably. However, strictly speaking pedigree analysis is a general term that refers to using pedigrees for genetic analysis. Pedigree analysis may include segregation analysis, linkage analysis and association analysis of candidate genes.

In segregation analyses of the periodontal diseases it is important to test multifactorial models as well as Mendelian models of major gene effects. Multifactorial models incorporate environmental and/or additional genetic factors, which may modify the clinical expression of disease in susceptible individuals. Finally, linkage studies can be used to confirm some genetic predisposition to disease and to identify risk genes. Linkage studies combine detailed clinical analyses of family members with genome wide scans, using known genetic markers to search for susceptibility or linked genes. Linkage is the association within families of two or more non-allelic genes resulting from their proximity on the same chromosome.

1.8.3 Prepubertal Periodontitis

As has been mentioned earlier, Page and colleagues have proposed that the generalised form of PP is an oral manifestation of the leucocyte adhesion deficiency syndromes (Page *et al.*, 1983; Page, Beatty and Waldrop, 1987; Meyle, 1994) (section 1.5.3.1). However, both GPP and LPP have also been described in otherwise healthy children (Butler, 1969; Fourel, 1972; Shapira, Schlesinger and Bimstein, 1997).

It has been proposed that PP is transmitted through an autosomal dominant mode of inheritance (Shapira, Schlesinger and Bimstein, 1997; Bimstein, Sela and Shapira, 1997). In one study of a large kindred, affected children were otherwise healthy and expressed normal levels of lymphocyte surface receptors including the adhesion molecules CD18 and CD11a. The occurrence of both GPP and LPP in siblings of this kindred suggests a common genetic aetiology for these disease forms. The differential expression of the phenotype could be due to the interaction of multiple genes or to the effects of interaction between the genotype and the environment.

PP has also been described in the offspring of a consanguineous marriage, which suggests that the disease may be transmitted by recessive genes (Lopez, 1992). In this report, both parents had AP. Two daughters, aged 13 and 15, had LEOP. Two other daughters and a son were diagnosed as having GPP, while two remaining siblings were not affected. Again, the co-occurrence of disease in one family points towards a common underlying genetic aetiology. In this family, GPP patients were otherwise healthy, while family members with LEOP, AP and one unaffected sibling showed depressed PMN chemotaxis (Lopez, 1992). Depressed neutrophil chemotaxis in GPP patients have been reported by others (Firatli *et al.*, 1996a), suggesting that the genetic determinants of neutrophil functions may affect risk for EOP.

1.8.3.1 Monogenic syndromes

GPP has often been reported in patients with certain monogenic syndromes. These conditions are rare, and may be divided into those manifesting defects in neutrophil number or function, and those with epithelial or connective tissue pathogeneses (Table 1.2).

Table 1.2 Inheritance of the monogenic syndromes associated with a high incidence of periodontal disease.

Taken from McKusick (1994) - Mendelian Inheritance in Man. A Catalog of Human Genes and Genetic Disorders.

Neutrophil Defects	**Inherited Trait**
Cyclic and Chronic Neutropenia	AD
Leucocyte Adhesion Deficiency	AR
Chediak-Higashi Syndrome	AR
Glycogen Storage Disease 1b	AR
Infantile Genetic Agranulocytosis	AR
Cohen's Syndrome	AR
Epithelial / Connective Tissue Defects	
Papillon-Lefèvre Syndrome	AR
Haim-Munk Syndrome	AR
Ehlers-Danlos Syndrome, Types IV and VIII	AD

Polymorphonuclear leucocytes are the first line of defence against infection. Defects in either their number or function can have profound effects on host susceptibility. For example, cyclic and chronic familial neutropenias are inherited as autosomal dominant traits. Children suffering from these conditions have an increased susceptibility to infection due to a decrease in the number of circulating neutrophils. They suffer from oral ulceration, angular stomatitis and severe periodontitis (Cohen and Morris, 1961; Andrews *et al.*, 1965; Kyle and Linman, 1970; Deasy *et al.*, 1980; Porter *et al.*, 1994). Variations in disease within the same family are thought to be due to differences in the standard of oral hygiene (Deasy *et al.*, 1980).

Leucocyte adhesion deficiency occurs in two forms (type I and type II), which are both inherited in an autosomal recessive manner. Although the numbers of circulating leucocytes may be elevated, cells have defective surface receptors and are unable to adhere to vascular endothelial cells. In LAD I the defect is in the CD11/CD18 family of receptors; in LAD II the sialyl Lewis-X receptor is abnormal. The anomalies in LAD I have been identified as various DNA coding irregularities, which may result in an abnormal three-dimensional assemblage of the receptors (Daniel and Van Dyke, 1996). Affected homozygotes suffer from acute recurrent infections, and those who survive infancy, develop severe periodontitis of the deciduous dentition immediately after eruption of the teeth (Waldrop *et al.*, 1987; Page, Beatty and Waldrop, 1987; Nagahata *et al.*, 1994; Paller *et al.*, 1994). In addition, examination of the root surfaces of teeth from children suffering from LAD I has revealed areas of hypoplastic cementum (Waldrop, Hallmon and Mealey, 1995). This defect may contribute to the manifestation of the aggressive form of periodontitis seen in these patients.

Chediak-Higashi syndrome and glycogen storage disease 1b are inherited as autosomal recessive traits associated with severe periodontitis (Tempel *et al.*, 1972; Hamilton and Giansanti, 1974; Charon, Mergenhagen and Gallin, 1985; Page and Beck, 1997). The chemotactic and bactericidal functions of neutrophils are abnormal in these patients. The faulty carbohydrate metabolism in individuals with glycogen storage disease 1b has been associated with low neutrophil numbers, impaired neutrophil function and periodontal disease (Hara *et al.*, 1987; Beaudet and Anderson, 1987).

Infantile genetic agranulocytosis (Kostmann syndrome) is a rare disorder, which is inherited in an autosomal recessive manner. This condition manifests as severe neutropenia and has also been associated with EOP (Kyle and Linman, 1970; Saglam *et al.*, 1995). Cohen's syndrome is another autosomal recessive syndrome and is characterised by non-progressive mental and motor retardation, infantile hypotonia, childhood obesity and numerous dysmorphic features. Neutropenia is also a feature of this condition. Individuals with Cohen's syndrome show more frequent and extensive BL than controls matched as far as possible for the degree of mental retardation (Alaluusua *et al.*, 1997). Cohen's syndrome patients also harbour one or more recognised periodontal pathogens in their subgingival plaque.

The most extensively studied of the monogenic conditions is the Papillon-Lefèvre syndrome (PLS). This disease will be discussed in some detail here, as a number of recent papers in the literature have improved our understanding of the genetic background to this condition. The classical clinical manifestations of PLS are hyperkeratotic lesions of the skin, affecting the palmoplantar surfaces, and EOP, affecting the deciduous and permanent dentitions (Papillon and Lefèvre, 1924). More recently, the Haim-Munk syndrome has been described. This condition is similar to PLS with the addition of arachnodactyly and a peculiar deformity of the terminal phalanges (Haim and Munk, 1965; Puliyel and Sridharan Iyer, 1986; Hart *et al.*, 1997). The clinical presentation of PLS can vary. Cases have been reported with no history of periodontitis of the deciduous dentition (Schroeder *et al.*, 1983; Willet *et al.*, 1985; Brown *et al.*, 1993; Bullon *et al.*, 1993; Soskolne *et al.*, 1996). Varying degrees of severity of periodontitis and abnormal keratosis have been noted (Soskolne *et al.*, 1996).

The underlying aetiology of PLS has been the subject of considerable debate in the literature (Gorlin, Sedano and Anderson, 1964; Shoshan, Finkelstein and Rosenzweig, 1970; Lyberg, 1982; Page and Baab, 1985; Preus, 1988). Defects in epithelium formation have been proposed (Gorlin, Sedano and Anderson, 1964; Lyberg, 1982; Page and Schroeder, 1982; Schroeder *et al.*, 1983). In addition, reduced lymphocyte response *in vitro* (Haneke, Hornstein and Lex, 1975; Levo, Wollner and Hacham-Zadeh, 1980) and decreased chemotaxis and phagocytosis of neutrophils and

monocytes have been suggested to cause the clinical manifestations seen in PLS patients (Djawari, 1978). These immunological defects may also explain the increased susceptibility to infections of patients with this syndrome (Haneke, Hornstein and Lex, 1975; Shams El Din *et al.*, 1984; Van Dyke *et al.*, 1984; Rateitschak-Plüss and Schroeder, 1984; Tinanoff *et al.*, 1986; Bullon *et al.*, 1993).

The Papillon-Lefèvre and Haim-Munk syndromes are inherited as autosomal recessive traits (McKusick, 1994). A high degree of consanguinity has been reported in families with these conditions (Pareek and Al Aska, 1986; Soskolne *et al.*, 1996; Hart *et al.*, 1997; Laass *et al.*, 1997; Fisher *et al.*, 1997). Recently, three studies involving genome wide linkage scans of consanguineous families linked the gene for PLS with a marker on the long arm of chromosome 11, near to the metalloproteinase gene cluster (Laass *et al.*, 1997; Fisher *et al.*, 1997; Hart *et al.*, 1998). These reports have identified additional loci for the palmoplantar keratodermas. Previously, several forms of these conditions had been linked with mutations in the two clusters of genes for the type I and type II keratin proteins, on the long arms of chromosomes 17 and 12 (Rogaev *et al.*, 1993; Lind *et al.*, 1994; Reis *et al.*, 1994). Cells of the epidermis become progressively impregnated with keratin, as the continuously dividing cells of the germinative layer push them towards the surface. It has been suggested that junctional epithelium shows a different type of expression of cytokeratins from the surrounding epithelium (Mackenzie *et al.*, 1991). Furthermore, it has been proposed that the development of junctional epithelium and palmoplantar epithelium may be affected by a common gene defect (Hart *et al.*, 1997). It now seems unlikely that this is the case following the confirmation of linkage between PLS and a marker on chromosome 11, separate from the gene clusters for the keratin proteins. Separate loci for palmoplantar hyperkeratosis and periodontitis, in linkage disequilibrium with one another, may be one explanation for the genetic basis of these clinical findings. This explanation has been previously proposed for LEOP and dentinogenesis imperfecta (Boughman *et al.*, 1986). It is also possible that the variation in the severity of the clinical features of PLS is due to the effects of two major genes, with other modifying genes influencing them in an additive or epistatic manner.

Many conditions and syndromes have been documented which are known to arise from different genes yet present with the same clinical manifestations (McKusick, 1994). It now seems clear that the early-onset periodontal diseases are genetically heterogeneous in nature (Potter, 1989; Sofaer, 1990; Hart, 1996). The three research groups who recently linked the gene for PLS with chromosome 11 may yet provide the first confirmation of an autosomal recessive form of early-onset periodontitis (Laass *et al.*, 1997; Fisher *et al.*, 1997; Hart *et al.*, 1998).

Ehlers-Danlos syndrome covers a group of connective tissue disorders that are characterised by a defect of collagen synthesis. Individuals with types IV and VIII inherit this defect in an autosomal dominant manner and have an increased susceptibility to periodontitis (Linch and Acton, 1979; Hartsfield, Jr. and Kousseff, 1990; Genco and Löe, 1993; Page and Beck, 1997). Clinical characteristics of type VIII Ehlers-Danlos syndrome include fragility of the oral mucosa and blood vessels and a severe form of EOP (Apaydin, 1995).

Other hereditary conditions that may reveal an associated periodontitis have been less well documented. These include the enzyme and enzyme inhibitor defects acatalasia and alpha-1-antitrypsin deficiency, which are autosomal dominant traits, and congenital erythropoietic porphyria, which is an autosomal recessive trait (Page and Beck, 1997). Alopecia, epilepsy and mental retardation are all inherited in an autosomal dominant manner (Page and Beck, 1997). Early-onset periodontitis has been reported in association with Weary-Kindler syndrome. Clinical manifestations of this condition also include epidermolysis bullosa and poikiloderma congenitale. The mode of inheritance of this rare syndrome is uncertain (Wiebe, Silver and Larjava, 1996).

It has been suggested that PP occurs only in children suffering from hypophosphatasia with an associated defective cementum (Baab *et al.*, 1986). This theory is difficult to accept. In individuals with hypophosphatasia, the premature loss of the primary teeth soon after their eruption into the mouth is not necessarily the result of a microbial infection and subsequent destruction of the periodontium. The condition is inherited in an autosomal dominant manner and is characterised by hypoplasia or aplasia of

cementum, which prevents the normal attachment of periodontal ligament fibres (Page and Beck, 1997). In a recent study, the histological appearance of the root surfaces of primary teeth was described in children with PP and no systemic disease (Bimstein *et al.*, 1998). It was found that some features of hypophosphatasia, such as atypical root resorption and the composition of the associated bacterial plaque, were also common to PP. However, there was no evidence of cementum aplasia which is characteristic of hypophosphatasia; instead, only abnormalities in cementum width were reported.

1.8.4 Localised and Generalised Early Onset Periodontitis

1.8.4.1 Inherited diseases and genetic syndromes

The incidence of periodontitis is higher in teenagers and adults with insulin dependent (type I) diabetes mellitus (Glavind, Lund and Löe, 1968; Cianciola *et al.*, 1982; Löe, 1993; Firatli, 1997). Type I diabetes mellitus is assumed to be autoimmune in nature and has been shown to have a clear genetic susceptibility (Todd, Bell and McDevitt, 1988; Davies *et al.*, 1994). Despite the association of periodontal disease and type I diabetes mellitus, no link between periodontitis and risk genes for diabetes has been established (Alley *et al.*, 1993; Gustke *et al.*, 1998).

The monogenic syndromes have been discussed in detail in the section on prepubertal periodontitis. In many of these conditions the permanent dentition is also affected to a greater or lesser extent. However, as has been mentioned earlier, most studies do not differentiate between the various forms of EOP. The periodontitis affecting the permanent dentition in Papillon-Lefèvre syndrome may appear as an acute severe generalised infection from the time of eruption, which is refractory to treatment (Haneke, Hornstein and Lex, 1975; Schroeder *et al.*, 1983; Shams El Din *et al.*, 1984; Van Dyke *et al.*, 1984; Rateitschak-Plüss and Schroeder, 1984; Çelenligil *et al.*, 1992; Soskolne *et al.*, 1996). Alternatively, it may manifest as a late-onset form of more moderate periodontitis (Willet *et al.*, 1985; Brown *et al.*, 1993; Fardal, Drangsholt and Olsen, 1998). Some cases respond to periodontal therapy and resolution of symptoms occurs (Tinanoff *et al.*, 1986; Preus and Gjermo, 1987; Ishikawa, Umeda and Laosrisin, 1994).

The frequency of infections in patients suffering from LAD I markedly decreases by the time they reach their late teens. It appears that other aspects of the immune response mature and compensate for the defective neutrophil function (Page *et al.*, 1997).

Trisomy 21 is a chromosomal aberration that should also be included here. Individuals with Down's syndrome have a higher incidence of periodontal disease, after taking into account levels of oral hygiene and other environmental variables (Cutress, 1971; Orner, 1976; Saxén, Aula and Westermarck, 1977; Saxén and Aula, 1982; Genco and Löe, 1993). In this condition extra copies of normal genetic material may result in aberrant gene dosage. The majority of cases of Down's syndrome are trisomic for the whole of chromosome 21. This chromosome contains genes encoding a number of functional and structural proteins (Sofaer, 1990). The Down's syndrome phenotype has, however, also been found with trisomy of band 21q22.3 alone (Pellissier *et al.*, 1988). It seems likely that the increased susceptibility to periodontal disease seen in patients with this syndrome, is caused by trisomy of another part of the chromosome, rather than being associated specifically with trisomy of band 21q22.3. One study has found decreased neutrophil chemotaxis in Down's subjects (Izumi *et al.*, 1989). Patients with Down's syndrome also suffer from frequent upper respiratory tract infections, indicating an immune defect in resistance to bacterial infection (Hassell and Harris, 1995). The gene directing synthesis of the beta subunit of the leucocyte adhesion molecule is found on chromosome 21 (McKusick, 1994), and may influence neutrophil chemotaxis in these patients.

1.8.4.2 Family studies

Many reports in the literature have documented the familial pattern of the early-onset periodontal diseases. In a recent North American survey of 7447 dentate individuals aged 13 years and older, the prevalence of EOP was low in younger age groups (Brown, Brunelle and Kingman, 1996). The prevalence of loss of attachment $\geq$ 5 mm was 0.3% in the 13-17 and 18-24 year olds, and 6.4% in the 25-34 year olds in this racially mixed population. In contrast, 40-50% of siblings in families with EOP may be similarly affected (Hart, 1996). This marked aggregation of EOP within families is

consistent with a genetic predisposition to this disease. However, familial patterns of disease may reflect not only a common genetic background but also exposure to common environmental factors (Hassell and Harris, 1995).

When evaluating the familial risk for periodontitis it is important to consider the myriad of known environmental and behavioural risk factors, including oral hygiene, exposure to specific oral bacteria, the presence of systemic disease, the competence of the immune system, smoking and psychological factors such as stress. Some of these factors themselves may be under genetic control; for example, the intelligence quotient of family members may influence oral hygiene standards.

The complex interactions between genes and the environment must also be considered when evaluating familial risk for the periodontal diseases. Currently, smoking and the presence of specific periodontal pathogens in subgingival plaque, appear to be the most important environmental risk factors for periodontitis (Bergström and Floderus-Myrhed, 1983; Grossi *et al.*, 1994; 1995; Page and Beck, 1997). Transmission of periodontal pathogens within families has been documented (Gunsolley *et al.*, 1990; Alaluusua, Asikainen and Lai, 1991; Petit *et al.*, 1993a; 1993b; Asikainen, Chen and Slots, 1996; Zambon *et al.*, 1996b; Stabholz *et al.*, 1998). A recent study by Stabholz *et al.* (1998) on a unique population of teenagers attending the same orthodox religious school near Jerusalem reported a high prevalence of LEOP. Ten of 15 affected families had more than one affected sibling. Following repeated and intensive questioning, only two sets of families were found to be related. However, because the 15 families were all of the same religion there were close social ties between them. The authors concluded that a strong environmental aetiology exists for LEOP. The findings of this study are in contrast to the consensus of the current literature. Further microbiological and linkage studies of this population should help to elucidate the contribution of genes and environment to the form of LEOP seen in these children. Since 75% of the parents originated from the East Coast of America and share the same orthodox religious beliefs, it is possible they may be unaware of being distantly related.

The observation of bacterial transmission within families is insufficient on its own to account for the familial clustering seen in EOP (Boughman, Astemborski and Suzuki, 1992; van der Velden *et al.*, 1993; Tinoco, Sivakumar and Preus, 1998). In addition, it is not clear whether the presence of specific periodontal pathogens is necessary to cause destruction in genetically susceptible individuals. It is possible that commensal bacteria in an individual with a deficient immune response may be sufficient to cause tissue damage (Hart, 1996). Environmental conditions in the periodontal pocket might then favour the growth of opportunistic or exogenous pathogens (van Steenbergen *et al.*, 1993). For example, smoking has important effects on the inflammatory and immune response (Kenney *et al.*, 1977; Kraal *et al.*, 1977; Totti *et al.*, 1984; MacFarlane *et al.*, 1992; Wolff, Dahlén and Aeppli, 1994; Tew *et al.*, 1996). Smoking may tip the balance towards periodontal destruction in a susceptible host by, for example, suppressing levels of IgG2 antibody specific for certain periodontal pathogens (Tew *et al.*, 1996). There is also evidence to suggest that smoking may alter the composition of subgingival plaque (Zambon *et al.*, 1996a).

Attempts to elucidate the role of inheritance in periodontal disease are complicated by the heterogeneous nature of these diseases. Variations in ages of onset and clinical presentations of the disease further complicate formal genetic studies. Finally, even within families, multiple forms of disease can co-exist (Spektor, Vandesteen and Page, 1985; Lopez, 1992; Boughman, Astemborski and Suzuki, 1992). Generalised prepubertal periodontitis, LEOP and AP can occur in the same family (Melnick, Shields and Bixler, 1976; Spektor, Vandesteen and Page, 1985; Lopez, 1992). Other reports have documented the sequential appearance of GPP, LEOP, and/or GEOP in the same subject (Butler, 1969; Jorgenson *et al.*, 1975; Shapira *et al.*, 1994a). It appears likely, therefore, that the various forms of EOP (PP, LEOP and GEOP), and possibly AP as well, have a common underlying genetic background. For the purposes of segregation analyses, most investigators have grouped the various forms of EOP together.

1.8.4.3 Segregation analyses

There are a number of obstacles inherent in genetic studies of EOP, not least of which is the difficulty in accurately diagnosing family members. Boughman *et al.* (1988) discussed this and other problems including the following findings: 1) EOP has a variable age of onset and in most cases is not recognised until after puberty; 2) the upper age limit of expression of the disease is curtailed, because loss of attachment in patients older than 35 years cannot always be differentiated from AP; and 3) the difficulty in accurately determining phenotypes of edentulous family members. Despite these difficulties, pedigree and segregation analyses of EOP support the role of a major gene. Various modes of inheritance have been postulated for EOP. These include X-linked dominant (Melnick, Shields and Bixler, 1976; Fretwell, Leinbach and Wiley, 1982; Page *et al.*, 1985; Spektor, Vandesteen and Page, 1985), autosomal recessive (Saxén, 1980b; Saxén and Nevanlinna, 1984; Beaty *et al.*, 1987; Long *et al.*, 1987; Boughman *et al.*, 1988) and autosomal dominant (Boughman *et al.*, 1986; Marazita *et al.*, 1994; Shapira, Schlesinger and Bimstein, 1997; Obach and Palomino, 1997). Most family studies of EOP have been conducted in North America and have consisted primarily of black families (Hart *et al.*, 1993; Marazita *et al.*, 1994).

Melnick and colleagues (1976) presented the most complete analysis of data from family studies found in the dental literature to date. Data from 19 sibships suggested that EOP was an X-linked dominant trait with reduced penetrance. Three important observations were made in support of this conclusion. Firstly, the observed ratio of females to males among affected persons was approximately 2:1. Secondly, no father-to-son transmission was seen. Finally, the segregation ratio of 0.39 was consistent with dominant transmission with reduced penetrance. The conclusion of this report was supported by two other studies of large families with a high prevalence of early-onset periodontitis (Page *et al.*, 1985; Spektor, Vandesteen and Page, 1985). However Spektor *et al.* (1985) noted that the disease prevalence in the immediate family was found to be unusually high for an assumed X-linked dominant trait.

Other researchers have disputed these conclusions. Hart *et al.* (1992) re-evaluated the evidence for X-linked dominant inheritance of EOP. They argued that early reports

were subject to proband bias because females are more likely to seek dental care than males (Benjamin and Baer, 1967). The proband is the affected individual through whom the family is identified. Two other papers found a predominance of female probands but equal sex distribution among affected relatives (Saxén and Nevanlinna, 1984; Hart *et al.*, 1991). The reported lack of father-to-son transmission of the disease was suggested to be due to incomplete pedigree information, because fathers were often not examined in family studies. Boughman *et al.* (1986) clearly demonstrated father-to-son transmission for EOP. Lastly, the segregation ratio of 0.39 is equally compatible with either X-linked or autosomal inheritance.

Saxen (1980b) examined 31 families and concluded that most probably EOP was inherited as an autosomal recessive trait with high penetrance in homozygotes. This was confirmed in a further study of 30 families (Saxén and Nevanlinna, 1984). Long *et al.* (1987) compared and evaluated autosomal recessive and X-linked dominant (reduced penetrance) modes of inheritance for LEOP and GEOP. They examined 33 families and concluded that the autosomal recessive model was far more likely than the X-linked dominant one. They did not, however, test for autosomal dominance, and only four families had multiple generations affected including parent-offspring pairs. This low frequency of vertical transmission favours recessive inheritance of this disease. This group also noticed that both LEOP and GEOP occurred together in several families, indicating a shared genetic predisposition (Long *et al.*, 1987).

An autosomal recessive model for EOP was proposed following the analysis of 28 kindreds (Beaty *et al.*, 1987; Boughman *et al.*, 1988). The effect of including age-limits in the diagnostic assessment of EOP was investigated. In order to reveal the likelihood of mistaking an autosomal dominant mode of inheritance for an autosomal recessive one, phenotypes were simulated for the 28 families according to an autosomal dominant model. All phenotypes of individuals under 12 years or over 35 years of age were designated as unknown. Simulations were then performed in a model-choice analysis using samples of the same size. The authors noted that the dominant model was chosen 60 of 100 times. The high rate of Type II error demonstrates the problems resulting from the age restrictions. Since familial clustering might be due to some shared environmental factors the authors tested a

sporadic model, which assumed that the distribution of disease was due to ubiquitous exposure to an environmental agent. The sporadic model was rejected in favour of a genetic model. A combination of genetic and environmental factors might indicate a general multifactorial model, but the observed risk to relatives (18.6% of total first-degree relatives were affected in the families studied) exceeded the expected proportion of 7.1% by a wide margin. The authors concluded that the autosomal recessive model was the preferred one, since it was not significantly better than a general autosomal model. To help overcome the problem of truncation of the affected phenotype, it has been suggested that more complete phenotypic information should be obtained for all adult relatives during the years of risk (Beaty *et al.*, 1987; Boughman *et al.*, 1988).

The most convincing evidence for autosomal dominant inheritance and race-specific heterogeneity in EOP was provided by Marazita *et al.* (1994). This large-scale segregation analysis included 100 nuclear families ascertained through 104 probands with EOP. Because many of the probands' relatives were over 35 years of age at the time of study, diagnoses of EOP were made using previous radiographs and dental histories. Progression from the localised to the generalised form of the disease was noted in 26 GEOP subjects. Sixteen families demonstrated co-occurrence of LEOP and GEOP. Although 100% fewer fathers than mothers took part in the study, the proportions of affected females to affected males was not significantly different among relatives of the probands (Hart *et al.*, 1991). The authors concluded that the most likely mode of inheritance was autosomal dominant in both black and non-black kindreds with 70% penetrance in black and 73% in non-black. They also detected significant racial heterogeneity for most parameter estimates.

A recent segregation analysis of families with EOP investigated the possibility of Mendelian inheritance of the IgG2 response of relatives (Marazita *et al.*, 1996). IgG2 levels were determined in 123 affected families and in 508 unrelated unaffected controls. The results of the segregation analysis indicated a major autosomal co-dominant mode of transmission for IgG2 levels, after adjusting for age, sex, race and LEOP status. Sixty-two percent of the variance in IgG2 levels was estimated to be due to the major locus, with significant spouse and parent/offspring residual correlations.

When smoking was also investigated as a covariate in a subset of 89 families, the best fitting model was still an autosomal co-dominant major locus with no residual correlations. This result suggests that the residual correlations in the first dataset were due to smoking habits.

1.8.4.4 Linkage studies

Very few family linkage studies of periodontitis have been undertaken. Two of these have been in families with a high prevalence of LEOP. Boughman *et al.* (1986) identified an autosomal dominant form of LEOP, in a large five-generation kindred from the Brandywine isolate in southern Maryland, and carried out a linkage study. It was found that both type III dentinogenesis imperfecta (DGI) and a localised form of EOP were segregating as dominant traits. In this study the gene for LEOP was linked to the vitamin D-binding protein group-specific component (Gc) locus, situated on the long arm of chromosome 4. The locus for the more common type of dentinogenesis imperfecta (DGI-II) had previously been linked with Gc. Linkage analysis of genetic and chromosomal markers on chromosome 4, suggested the gene order to be 4cen--LEOP--Gc--DGI--MNS--qter (Cohen *et al.*, 1986).

Another group re-evaluated the above finding by studying 19 unrelated families, each with two or more EOP affected individuals (Hart *et al.*, 1993). Their results indicated no significant evidence of linkage to this region for any of the models tested. It was hypothesised that this could be due to a previous false positive report of linkage. Alternatively, it was suggested that genetic locus heterogeneity might exist in EOP. This could be due to racial differences or because the Brandywine family had a form of LEOP that differed from the type found in the families in the subsequent study.

A large scale linkage study of 100 families with EOP also did not confirm linkage to chromosome 4 (Wang *et al.*, 1996; Hart and Kornman, 1997). Linkage was found between susceptibility to EOP and the HLA region of chromosome six and the region coding for the COX-1 enzyme system on the long arm of chromosome 9. COX-1 is involved in synthesis of PGE_2 and may be important in regulating levels of this inflammatory mediator in periodontal disease.

1.8.4.5 Association studies

A study, in a population of Japanese patients, found a significant association between an atypical *Bam*HI restriction site in the *HLADQB* gene and EOP (Takashiba *et al.*, 1994). More recently a French group investigated *HLADR* polymorphisms in patients with rapidly progressive disease (Bonfil *et al.*, 1999). They found a significant association between alleles *DRB1*0401, 0404, 0405, 0408* and GEOP. These alleles had previously been found to be associated with RA.

A recent study in North America found very significant evidence of linkage disequilibrium between allele one of the *IL1A –889* gene and allele one of the *IL1B +3953* gene and GEOP (Diehl *et al.*, 1999) (section 1.8.5.3). A number of multiplex families were used to investigate these candidate genes in EOP. In this report, both the transmission disequilibrium test (TDT) and the affected sib pair design were used to examine the association between the two *IL1* polymorphisms. The TDT test compares the number of transmissions of specific alleles from heterozygous parents to affected offspring. For GEOP subjects allele one of the *IL1A –889* polymorphism was transmitted significantly more frequently than allele two ($p = 0.0065$). There were seventeen transmissions of allele one of the *IL1B +3953* gene and only two transmissions of allele two ($p = 0.0005$). Similar but non-significant trends were found for LEOP. Associations were observed in both smokers and non-smokers. The homozygous (1,1) *IL1B +3953* genotype was also transmitted significantly more often to GEOP affected individuals than to unaffected subjects ($p = 0.014$). These findings were consistent in both African Americans and Caucasians, although the numbers in some categories were small. Further analyses revealed that the transmission disequilibrium is more strongly associated with the *IL1B +3953* polymorphism. These results provide evidence that the *IL1B +3953* polymorphism (allele one) may be in linkage disequilbrium with one EOP gene. Results from the affected sib pair analysis were less compelling.

1.8.5 Adult Periodontitis

1.8.5.1 Monogenic syndromes

Varying degrees of periodontitis of the permanent dentition have been reported for some of the monogenic syndromes. In cyclic and chronic neutropenia, the neutropenia and associated periodontitis may be less severe in the permanent than in the deciduous dentition (Sofaer, 1990). In heterozygote individuals with LAD, who have half the amount of normal membrane glycoprotein in the cell surface receptors, less severe BL of the permanent dentition may be evident later in life (Waldrop *et al.*, 1987). It is possible that there is coincidental development of AP, which is not related to LAD.

1.8.5.2 Twin studies

Despite the twin model being a very powerful method of providing evidence of a genetic predisposition to disease, there have been very few twin studies of AP. An early study, which investigated levels of gingival recession and crevice depth, and indices of gingivitis, supragingival plaque and calculus, in 26 twin pairs aged 12-17 years, found no evidence of genetic influence (Ciancio, Hazen and Cunat, 1969). An explanation for this finding may have been the relatively young age of the individuals and the small sample size.

More recently, larger studies of older subjects have found an association between genetic variation and individual susceptibility to AP. Corey *et al.* (1993) collected questionnaire data of periodontal health from 4,908 twin pairs included in the population-based Virginia Twin Registry. Four hundred and twenty individuals (mean age 31.4±0.7 years), who were members of 116 identical and 233 fraternal twin pairs, reported a history of periodontitis. The concordance rate ranged from 0.23 to 0.38 for MZ twins, and 0.08 to 0.16 for DZ twins. It was concluded from these results that adult-onset periodontal disease has a genetic susceptibility. However, in this study no data on smoking status were collected for the subjects who took part and no adjustments were made for sex and age, which may inflate twin correlation (McGue and Bouchard, 1984).

A large-scale study of twins recruited from the Minnesota Twin Registry and the Minnesota Study of Twins Reared-Apart was undertaken by Michalowicz and co-workers (Michalowicz *et al.*, 1991a; 1991b; Michalowicz, 1994; Rudney *et al.*, 1994). They investigated clinical status and host risk factors for periodontal disease in 17 pairs of reared-apart dizygous twins (DZA), 63 pairs of dizygous twins reared-together (DZT), 83 pairs of monozygous twins reared-together (MZT) and 21 pairs of monozygous twins reared-apart (MZA). Ratio of the within-pair variances of dizygous to monozygous twins was significantly greater than zero for mean probing depth and AL, for both unadjusted and adjusted values. It is important to bear in mind that the majority of twins in this study had mild AL. Therefore, it is possible that the small variance in AL, with a concurrent large measurement error, might mask an even greater genetic contribution. Alternatively, more severe disease may show greater or less genetic variability. The correlation for AL was similar between MZT and MZA twin groups, which indicates that a common environment does not influence AL in adults. In addition, MZT twins were found to be less concordant for oral hygiene habits than DZT twins. Therefore, the mean probing depth and attachment level scores were found to vary less for MZT than for DZT twin pairs, despite the reverse being the case for oral hygiene practices. Investigation of alveolar bone height in twins from the Minesota study also showed significant variation due to differences in genotype (Michalowicz *et al.*, 1991b). Subgingival plaque samples from 169 twin pairs were also analysed for the presence of Pi, Pg, Aa, Ec and Fn using an immunoassay (Michalowicz *et al.*, 1999). For all species examined, the concordance rates were not significantly different between MZ and DZ twin groups. These findings were apparent despite similar smoking histories, self-reported oral hygiene practices and antibiotic use in the twin groups. It was concluded that there is a genetic contribution to clinical and radiographical, but not microbiological, measures of disease. However, the mode of transmission of the trait may be a complex interaction between alleles at more than one locus, or between genetic and environmental and/or systemic factors.

Studies of structural or immunological features, which may have an effect on expression of periodontal disease, have been investigated in twins. Examination of salivary protein levels, in monozygous and dizygous twins, found the correlation for total protein, lactoferrin and total peroxidase indicative of a shared genetic and

environmental model. However, a much more complicated genetic influence was suggested by data for myeloperoxidase (Michalowicz *et al.*, 1991b). *In vitro* models of cells cultured from twins have shown that proliferation of fibroblasts from gingival biopsies of identical and fraternal twins are under significant genetic influence (Cockey *et al.*, 1989; Hassell and Harris, 1995). In addition, the exposure of fibroblasts to extracts from periodontal pathogens has found a strong genetic susceptibility to these bacteria. This is manifested in the cytoskeletal structure of these cells (Hassell and Harris, 1995; Hassell *et al.*, 1997).

1.8.5.3 Association studies

Two studies of large populations of patients and race-matched controls, have recently investigated associations between Fc-gamma receptor (FcγR) polymorphisms and AP (Kobayashi *et al.*, 1997; Van Schie *et al.*, 1998) (section 1.8.6.3). *FcγRIIa* and *FcγRIIIb* genotypes were investigated in 100 Japanese maintenance patients and 105 healthy controls (Kobayashi *et al.*, 1997). No associations were found between any specific genotype and the disease. However, in those patients with recurrence of disease the *FcγRIIIbNA2* allele was found significantly more frequently ($p < 0.05$). Furthermore, the annual rate of recurrence was significantly higher in patients homozygous for *NA2* and heterozygous for *NA2/NA1* than in those who were homozygous for *NA1* ($p < 0.05$). It is interesting to note that only six patients were homozygous for the *FcγRIIaR131* genotype in this report and all of them showed recurrence of disease, although the results were not significant ($p = 0.06$). It has been demonstrated previously that a very low prevalence of the *FcγRIIaR/R131* genotype exists in Japanese individuals (Osborne *et al.*, 1994; Rascu *et al.*, 1997). This may account for the lack of a significant finding for this genotype. The authors also investigated whether the following confounding factors influenced the correlation between the *FcγRIIIbNA2* allele and disease recurrence: serum IgG subclass concentrations; clinical parameters at baseline and at follow-up; differences in the distribution of disease recurrence between mild and severe forms of AP; and smoking. None of these covariates were found to be responsible for the observed correlation. It was concluded that the *FcγRIIIbNA2* allele may be a risk factor for recurrence of

periodontitis. In addition, it was suggested that the association of the *FcγRIIaR/R131* genotype with disease recurrence should be investigated in other ethnic populations (Kobayashi *et al.*, 1997).

In another study in American Caucasians, 105 patients with moderate to severe AP and 132 age-matched and healthy controls were examined for *FcγRIIa* and *FcγRIIIb* genotypes (Van Schie *et al.*, 1998). *FcγRIIaH/H131* combined with *FcγRIIIbNA2/NA2* was found significantly more frequently in patients than controls. *FcγRIIaR/H131* combined with *FcγRIIIbNA2/NA2* was significantly reduced in patients compared with controls ($p = 0.035$). Neither genotype demonstrated any association when considered individually. These results are confusing in the light of the knowledge we have concerning the protective role of the *FcγRIIaH/H131* genotype (Sanders *et al.*, 1995; Wilson and Kalmar, 1996). It is possible that these combined genotypes are in linkage disequilibrium with other alleles and form part of an extended haplotype, rather than being directly responsible for immune defects themselves.

Colombo *et al.* (1998a) recently investigated serum levels of IgG2, Gm(23) allotype and *FcγRIIa* and *FcγRIIIb* genotypes in 32 refractory, 54 successfully treated and 27 periodontally healthy subjects. The number of smokers was not significantly different between the groups. No significant differences in serum IgG2 levels, Gm(23) allotypes or *FcγR* genotypes were found between the three groups. These variables were weakly related or not related to clinical status. It is possible that no relationship exists between any of these factors and periodontal disease. Alternatively the heterogeneous and multifactorial aetiology of the disease may mask any association, because of the varying importance of specific factors between individuals. In addition, the authors did point out that specificity of IgG2 was not determined, which might cause confusion because of raised IgG2 levels to an infection in a different body system.

A recent paper has indicated an association between a composite genotype of allele 2 of the *IL1B +3953* gene and allele 2 of the *IL1A -889* gene and the severe form of AP (Kornman *et al.*, 1997). All the patients in this study were Caucasian and of Northern European origin. The association was only found in non-smokers. Those non-

smokers who were positive for the composite genotype had the same risk of disease as smokers who were either genotype-positive or genotype-negative. This result requires corroboration in larger studies of AP patients. There were only eighteen patients in the severe non-smoking group and although the OR between severe versus mild AP was large (18.90), the confidence intervals were very wide (1.04-343.05).

Another recent report of a population of Caucasian AP patients, found an association between allele 2 of the *IL1B +3953* gene and advanced periodontitis. This genotype occurred more frequently in patients with advanced disease, when compared to patients with either mild or moderate disease (Gore *et al.*, 1998). However, there was no significant difference between the advanced group and the healthy control group with regard to this allele. No associations were found between the *IL1A -889* gene and any of the disease categories or between patients and healthy controls. Furthermore, an investigation of the composite genotype (allele 2 of the *IL1B +3953* gene and allele 2 of the *IL1A -889* gene (Kornman *et al.*, 1997)), demonstrated a very significant association ($p = 0.002$, 2-tail Fisher's exact test) between this genotype and both patient and control groups. These findings are difficult to interpret but do appear to bring into question the results of Kornman *et al.* (1997). However, the findings of the more recent study should also be treated with caution, as the numbers of individuals in the different categories of disease severity were small ($n = 10$ for mild, 10 for moderate, 12 for severe). Because of the small size of the groups, no analysis of the contribution of smoking as a confounding variable could be carried out (Gore *et al.*, 1998).

It is also interesting to note, that the findings of both of these groups differ from those of Diehl *et al.* (1998) who studied *IL1* polymorphisms in EOP (section 1.8.4.4). In the EOP study linkage disequilibrium was found between GEOP and allele 1 of the *IL1B +3953* gene ($p = 0.0005$). Allele one of *IL1A –899* was also transmitted significantly more frequently ($p = 0.0065$). This may be due to differences between diagnostic groups. However the latter study did investigate a much larger group of individuals. Twenty-eight African American families, with 94 EOP affected and 85 unaffected subjects, and seven Caucasian families, with 31 EOP affected and 36 unaffected subjects, were included in the analysis.

Galbraith *et al.* (1998) also determined *TNF* genotypes in the same populations of AP patients and healthy controls as were investigated for *IL1* polymorphisms (Gore *et al.*, 1998). TNFα production by oral PMN was also measured. No differences in the three-biallelic polymorphisms of *TNF* (*-238*, *-308*, *+252*) (Figure 4.1) were found between AP patients and controls, or between patients with different levels of disease severity. However, a significant association was noted between the level of TNFα production by oral PMN and the heterozygous *T1,2* genotype of *TNFA -308* ($p = 0.037$). When severity of disease was investigated in relation to level of secretion of TNFα, an association between the *T1,2* genotype and increased production of TNFα was only seen in patients with severe disease. Again the small sample size of the different disease categories should be noted. Furthermore only three patients in the severe group showed increased production of TNFα and were also heterozygous for the *TNFA -308* gene polymorphism.

1.8.6 Aspects of the Host Response which may form the basis of Genetic Susceptibility to EOP

The genetic basis of a number of aspects of the host response has already been discussed earlier. The aim of this section is to summarise the role of these innate, inflammatory and immunological defects.

There are several features of the host's innate immune system, which may contribute to genetic susceptibility to EOP. These include epithelial and connective tissue anomalies (section 1.8.3.1) and fibroblast defects (Cockey *et al.*, 1989; Anavi *et al.*, 1989; Hassell *et al.*, 1997; Aldred and Bartold, 1998). Other possible traits are alkaline phosphatase deficiency resulting in defective cementum (Baab *et al.*, 1986) and variation in salivary protein levels (Rudney *et al.*, 1994).

Defects in the inflammatory and humoral immune response which might predispose an individual to periodontitis are discussed below.

1.8.6.1 IgG2 response

Lu *et al.* (1994) have shown that serum IgG2 levels are increased in LEOP patients, as opposed to GEOP, AP or unaffected individuals. This point is of interest because IgG2 is the predominant immunoglobulin subclass reactive with Aa serotype b outer membrane antigens. Aa is a periodontal pathogen considered important in early-onset periodontitis (Wilson and Hamilton, 1992; Lu *et al.*, 1993; Wilson, Bronson and Hamilton, 1995). Serum IgG2 levels were shown to increase with age and there was a sharp rise at around puberty (Lu *et al.*, 1994). Black subjects in all diagnostic groups had higher levels of IgG2 than whites. The incidence of LEOP among black individuals has been shown to be at least 15 times greater than among whites (Saxby, 1987; Löe and Brown, 1991; Melvin, Sandifer and Gray, 1991). Furthermore, the increase in serum IgG2 levels in LEOP patients was found not to be entirely attributable to high anti-Aa serotype b levels which accounted for only about 10% of the excess IgG2 in LEOP patients' sera. It appears that LEOP patients, particularly black individuals, have a tendency, perhaps a genetic predisposition, to produce increased levels of IgG2 (Tew *et al.*, 1996).

A recent segregation analysis of families with EOP indicated a major autosomal co-dominant mode of transmission for IgG2 levels, after adjusting for age, sex, race and LEOP status (Marazita *et al.*, 1996) (section 1.8.4.3). Convincing evidence exists for a protective role of high levels of IgG2 in patients with LEOP (Tew *et al.*, 1996). Smoking has been found to suppress the IgG2 response in AP and GEOP patients and is associated with more severe destruction. This does not appear to occur in LEOP patients (Tew *et al.*, 1996). Furthermore, the lower IgG2 titres seen in black GEOP smokers may be specific to Aa. IgG2 response against antigens of other bacteria may not be lower in GEOP subjects who smoke (Tangada *et al.*, 1997).

Definition at the gene level for this major autosomal co-dominant trait for IgG2 response may lie within the major histocompatability complex on chromosome six (section 1.4).

1.8.6.2 Gm allotypes

Another region of the genome that could determine serum levels of IgG2 is the γ-2 heavy chain gene complex situated on chromosome 14, which influences Gm allotypes found on IgG molecules. Only one allotype has been found to be associated with IgG2 and this is Gm23 (G2m[n]) (Marazita *et al.*, 1996). It has been shown that one allele of the *Gm23* locus produces higher levels of IgG2 (Rautonen, Sarvas and Mäkelä, 1989). Furthermore, immune response genes within the *MHC* interact with *Gm*-linked genes in influencing serum antibody responses to specific bacterial antigens (Whittingham *et al.*, 1980). However, in the study of Marazita *et al.* (1996) segregation of Gm23 was not found to be responsible for the co-dominant mode of inheritance they observed. In addition, Colombo *et al.* (1998a) did not find any association between Gm allotypes and refractory periodontitis, successfully treated periodontitis or a healthy periodontium.

1.8.6.3 Fcγ receptors

Some investigators have shown that patients with GEOP can demonstrate high antibody titres against Aa serotype b outer membrane antigens (Gunsolley *et al.*, 1988; Wilson and Hamilton, 1995; Tew *et al.*, 1996). It has also been suggested that the subclass IgG2 is a poor opsonin because it fixes complement less effectively than IgG3 and IgG1 and binds weakly to Fc receptors on the surface of phagocytes (Roitt, 1994; Wilson and Kalmar, 1996). These observations complicate any rationalisation of the proposed protective effect of high anti-Aa titres of IgG2 in black LEOP patients. More recently, another aspect of the immune response has been elucidated that may explain this phenomenon.

Fc receptors provide a critical link between the humoral and cell-mediated components of the immune system. FcγR on phagocytes recognise the Fc region of IgG. An excellent review of the possible role of FcγR in determining susceptibility to LEOP was recently presented by Wilson and Kalmar (1996). What follows is a brief resumé. It has been shown that neutrophils express low-affinity FcγR (FcγRII [CD32] and FcγRIII [CD16]) under normal conditions. FcγRII bind all subclasses of IgG but FcγRIII only bind IgG3 and IgG1. Many more FcγRIII than FcγRII are expressed on

the surface of neutrophils. However, while FcγRIII can evoke lysosomal degranulation they are incapable of promoting respiratory burst activity and phagocytosis. FcγRII on the other hand can precipitate all three activities. It is possible that FcγRIII may prepare PMNs for phagocytosis mediated by FcγRII (Wilson and Kalmar, 1996).

It appears likely that phagocytosis of Aa opsonised with IgG2 is mediated by the presence of FcγRII. FcγRII and FcγRIII exist in a number of isoforms coded by genes situated on the long arm of chromosome 1. Both FcγRIIa and FcγRIIIb show biallelic polymorphism which have been shown to influence neutrophil phagocytic capacity (Kobayashi *et al.*, 1997). IgG2 is bound more effectively by the H131 allotype of FcγRIIa than by the R131 allotype and opsonised particles are phagocytosed more efficiently (Sanders *et al.*, 1995; Wilson and Kalmar, 1996). Thus the possibility exists that the perceived protective effect of a high IgG2 titre reactive against Aa is also dependent on the H131 allotype of FcγRIIa. A recent study found that opsonic activity of affinity-purified IgG2, reactive against cell-envelope antigens of Aa, from patients with LEOP but not from healthy sera effectively mediated phagocytosis and killing by neutrophils which were homozygous for the H131 allotype (Wilson, Bronson and Hamilton, 1995). Two earlier studies had found that in either the presence or absence of complement, opsonisation of Aa by IgG2 is enhanced by the presence of neutrophils that are homozygous for H131 and inhibited by neutrophils homozygous for R131 (Cutler, Kalmar and Arnold, 1991).

It has been reported that the numbers of FcγRII and FcγRIII on peripheral blood neutrophils of LEOP patients are within the normal range (Leino *et al.*, 1994). However, uncertainty exists regarding which allotype is present. Miyazaki *et al.* (1997) found that both FcγRII and FcγRIII expression and neutrophil phagocytosis were significantly depressed in GCF compared with peripheral blood in AP patients. The reduction in FcγR significantly correlated with phagocytic activity. In addition the mRNA level of FcγRIII was also significantly lower in GCF neutrophils than in peripheral blood neutrophils, but not the mRNA level of FcγRII. These findings indicate local suppression of FcγR expression and/or the production of Fcγ-binding proteins by periodontal pathogens (Hillestad, Helgeland and Tolo, 1996). In this study

individual FcγR allotypes were not examined. There have been no published reports of *FcγR* polymorphisms in LEOP or GEOP patients but three research groups have investigated *FcγR* polymorphisms in AP patients (Kobayashi *et al.*, 1997; Van Schie *et al.*, 1998; Colombo *et al.*, 1998a) (section 1.8.5.3). Research in the future aimed at defining which allotypes are present on both peripheral and gingival neutrophils in relation to genotype, in different categories of periodontal disease and different racial groups, may yield interesting results.

1.8.6.4 Neutrophil chemotactic defects

Another possible mechanism whereby genetic associations are translated into an increased susceptibility to EOP are through chemotactic defects of neutrophils. A high percentage of members of families with a background of LEOP show abnormal neutrophil chemotaxis (Van Dyke *et al.*, 1985). Analysis of 22 families with LEOP demonstrated that in 19 of these families the proband and all affected siblings manifested neutrophil chemotaxis disorders. In the families investigated the chemotactic defect was found in almost 50% of the siblings, indicating a dominant mode of inheritance (Van Dyke *et al.*, 1985). Whether the trait is due to an intrinsic defect of neutrophils or is caused by extrinsic factors in the sera is controversial (Agarwal *et al.*, 1996). If the neutrophil chemotactic defect is the manifestation of a major gene locus, heterogeneity of LEOP exists because a significant number of patients and families with LEOP do not show evidence of the defect (Kinane *et al.*, 1989; Page and Beck, 1997).

Reports of decreased neutrophil chemotaxis in LEOP have been associated with a lower number of receptors for chemoattractants such as N-formyl-methyl-leucyl-phenylalanine (FMLP), complement fragment C5a, leukotriene B4 and interleukin-8 (De Nardin *et al.*, 1990; Daniel and Van Dyke, 1996). A cell surface glycoprotein (gp110) possibly involved in neutrophil movement and secretion is reduced in LEOP patients (Van Dyke *et al.*, 1987). In addition, several other post-receptor defects have been identified (Daniel and Van Dyke, 1996). Recently De Nardin (1996) investigated decreased FMLP binding in relation to variations in FMLP receptor DNA from patients with LEOP and from healthy controls. Initial results indicated a base change

in the FMLP receptor DNA from LEOP patients with decreased FMLP binding. This resulted in a different amino acid in a region of the receptor molecule which has been shown to influence ligand binding. An LEOP patient and healthy controls with normal receptor binding showed no changes in DNA. These preliminary results indicate a possible role for a FMLP receptor alteration in neutrophil chemotaxis. However, the author points out in the discussion that many of the molecules related to neutrophil dysfunction in LEOP patients belong to the same family of surface receptors and have similar morphogenic characteristics. Similarities in signal transduction mechanism between cell surface components also exist. De Nardin (1996) hypothesised that a common underlying defect, at either the cell surface receptor or post-receptor level, contributed to the alteration in neutrophil function seen in patients with LEOP.

1.8.6.5 Inflammatory mediators

It has been demonstrated that sera from LEOP patients with decreased neutrophil function can modify the activity of healthy neutrophils (Agarwal *et al.*, 1996). The serum factors responsible for these effects have been identified as being inflammatory mediators such as prostaglandins and cytokines. Small quantities of bacterial lipopolysaccharide (12.5 pg/ml) can induce the secretion of TNFα, IL-1 and PGE_2 from monocytes. The production of IL-1-TNFα and TNFα-PGE_2 are strongly correlated (Mølvig *et al.*, 1988). Very small increases in serum cytokine levels can alter neutrophil function. PGE_2 and cytokines also stimulate fibroblasts to release MMPs, which break down the extracellular matrix. In addition, IL-1, TNFα and PGE_2 are potent stimulators of bone resorption. Adherent mononuclear cells from LEOP patients have been found to secrete higher levels of PGE_2 and TNFα than GEOP patients or healthy controls (Shapira *et al.*, 1994c). More recently, insulin-dependent diabetic patients with periodontitis have been reported to manifest excessive levels of inflammatory mediators (Salvi *et al.*, 1997a; 1997b; Salvi, Beck and Offenbacher, 1998). It has been suggested that these observations indicate an underlying immune defect of monocytes in certain diagnostic groups (Offenbacher, 1996). However, there are dissenters from this theory (Kjeldsen *et al.*, 1995; Shapira, Soskolne and Van Dyke, 1996).

A genetic basis for the concept of excessive production of inflammatory mediators by monocytes has recently begun to appear in the literature. Early investigations of genetic polymorphisms of cytokines have attempted to identify a possible link between different periodontal categories and specific alleles (Kornman *et al.*, 1997; Diehl *et al.*, 1998; Galbraith *et al.*, 1998; Gore *et al.*, 1998) (section 1.8.5.3). In addition, a family linkage study of EOP linked the disease with the region encoding the COX-1 enzyme system on the long arm of chromosome 9 (section 1.8.4.4). This system acts as a catalyst for the breakdown of arachidonic acid, one of the products of which is PGE_2 (Gemmell, Marshall and Seymour, 1997).

1.8.7 Summary

It is important to highlight the use of clear diagnostic criteria in the investigation of hereditary diseases. Genetic analyses must be underpinned by reliable clinical diagnoses within heterogeneous populations; otherwise genetic transmission assessments will be erroneous.

A number of aspects of the inflammatory and immune response that are suspected to play a role in the development of periodontal disease have a clearly defined genetic basis (Malo and Skamene, 1994; Wilson, di Giovine and Duff, 1995; Vyse and Todd, 1996). Possible associations between several of these aspects of immune defence and genotype have been discussed in the context of both family and population studies.

One hypothesis of the complex multifactorial background to EOP is that mutations exist that cause general defects in immune responsiveness, whereas other genes cause specific defects in the periodontium. The genes controlling immune regulation could interact in an additive, synergistic or epistatic manner with genes of major effect inherited in a Mendelian fashion (Hart, 1996). Furthermore, environmental factors might affect the phenotypic expression of the genotype of different individuals in a diverse manner. This explanation would allow for the variation in age of onset and extent and severity of disease seen in patients with EOP.

1.9 Aims and Objectives

1.9.1 Genetic Markers of Susceptibility to Periodontitis

As mentioned earlier, no host-based biochemical or cellular markers of periodontitis have so far been identified. Many candidate aetiopathogenic factors have been proposed as possible disease markers but no single entity has emerged as pathognomonic for any of the periodontal diseases. Several factors appear to be useful. However, doubts exist as to their role in aetiopathogenesis and considerable technical difficulties prevail in their application. Investigations of cytokine levels or the humoral response in serum, gingival tissue cell supernatants and GCF have produced conflicting results from different research groups. This is due to the following reasons: the reverse-transcriptase polymerase chain-reaction (RT-PCR) which is one method of measuring cytokine levels lacks quantitation (Gemmell and Seymour, 1998); because of the short half-life of some cytokine mRNAs, such as IL-4, measurements do not reflect actual cytokine production; large individual variation exists in antibody titres and avidities among patients and controls, there is very probably cross-reactivity between antigens of periodontal bacteria and those of the gastro-intestinal tract; during tissue remodelling, increased levels of both proinflammatory and anti-inflammatory cytokines are found; tissue breakdown products such as alkaline phosphatase and glycosaminoglycans can also be found during remodelling; and finally, bacterial proteases may breakdown antibodies and cytokines in the tissues and GCF. Assessing the numbers of immune and inflammatory cells and differences in their intracytoplasmic cytokine synthesis patterns in gingival tissues is probably more informative. Immunohistochemistry, *in situ* hybridisation and flow cytometry techniques are methods of investigating variations between diseased and healthy tissues and among different categories of periodontitis. However, due to wide individual variation and the dynamic nature of the disease process, no markers of disease status have yet come to light.

Considering the problems outlined above, the identification of a stable marker of periodontal disease susceptibility which is independent of the immune response would be appealing. Recently, researchers have investigated associations between genetic

markers and certain autoimmune and infectious conditions. Molecular biological techniques can be used to differentiate polymorphisms of candidate genes, which have been selected because their protein products play a role in the inflammatory or immune response. Two techniques employed to distinguish the different polymorphisms include PCR and RFLP analysis.

1.9.1.1 Genetic polymorphisms

A genetic polymorphism is the long-term occurrence in a population of two or more genotypes, which could not be maintained by recurrent mutation (Connor and Ferguson-Smith, 1997). Usually if one in fifty of the population have the rare genotype then the condition is polymorphic. Recent research has indicated that most genes are polymorphic. Variations in DNA may result in no change to the protein product if the polymorphism is found in a non-coding region of the genome (intron), or if they still code for the same amino acid. Alternatively, the polymorphic alleles may result in an altered protein product which affects the function of the protein and its electrophoretic mobility. Polymorphisms occur because of frequent selection of mutations in particular habitats. They may also arise as a result of random genetic drift.

1.9.1.2 Polymerase chain reaction

The PCR technique involves the amplification of a specific segment of DNA from the candidate gene under investigation. In the first step, genomic DNA is denatured by heating to 94°C. Two synthetic oligonucleotide primers anneal to their complementary coded sequences either side of the target segment on separate strands of DNA, at their optimum annealing temperature (usually 50-60°C). After annealing, the primers are extended at 72 °C, in the presence of deoxyribonucleotide triphosphates (dNTPs), a reaction catalysed by a thermostable DNA polymerase enzyme. The three stages of denaturation, annealing and extension constitute one cycle of PCR and the resulting product serves as a template for the next cycle. Further cycles result in exponential accumulation of the target sequence. The PCR product can then be digested using an

appropriate restriction enzyme and the fragments separated using gel electrophoresis and visualised under UV light when stained with ethidium bromide.

1.9.1.3 Restriction fragment length polymorphism analysis

Restriction enzymes are found in many bacteria and serve to prevent the incorporation of foreign DNA (Connor and Ferguson-Smith, 1997). More than 400 restriction enzymes have been identified and they are named after the bacterium which produces them. Each enzyme will only cleave at a particular recognition site, which is usually 4 to 6 base pairs in length. Digestion yields fragments with either blunt or sticky (staggered) ends. There are about 100 different recognition sites throughout the human genome. Restriction enzymes can be used to differentiate allelic polymorphisms, by the presence or absence of a specific recognition site.

1.9.1.4 Aims and objectives

Using the techniques described above the studies of candidate genes presented in this thesis sought to answer the following questions:

1. Is the *HLADQB1* polymorphism associated with GEOP in a Japanese population, also associated with the disease in a Caucasian population?

2. Is GEOP associated with specific polymorphisms in the *IL10G* and *IL10R* microsatellites?

3. Is GEOP associated with specific polymorphisms in the *TNFa* and *TNFd* microsatellites?

4. Are there any differences in the frequency of these polymorphisms between GEOP smokers and non-smokers?

1.9.2 Clinical and Genetic Analysis of GEOP

Many difficulties arise when studying delayed onset hereditary traits. In some conditions, such as Huntington's chorea and also in periodontal disease, the diagnosis

is only possible relatively late in life. The problems of genetic model testing in EOP have been highlighted previously (Boughman *et al.*, 1988): 1) EOP has a variable age of onset and in most cases is not recognised until after puberty; 2) the upper age limit of expression of the disease is curtailed, because loss of attachment in patients older than 35 years cannot always be differentiated from AP; and 3) it is difficult to obtain accurate phenotypic information for edentulous family members. These factors create substantial problems for genetic studies of EOP.

To help overcome the problem of truncation of the affected phenotype, it has been suggested that more complete phenotypic information should be obtained for all adult relatives during the years of risk (Beaty *et al.*, 1987; Boughman *et al.*, 1988).

1.9.2.1 Aims and objectives

The aim of the final study in this thesis was to develop a system in which documented clinical evidence and reported histories could be used to accurately diagnose untreated disease, in an equivalent manner to full periodontal chartings. This index could then be used for the current and retrospective diagnosis of EOP relatives. A method is presented which objectively addresses the difficulties encountered in defining affected and unaffected categories. Using this index, the results of the segregation analysis on an extended North European Caucasian kindred with 14 affected individuals is presented, to determine whether this family is suitable for a linkage study.

Chapter 2 General Materials and Methods

2.1 Introduction

The following chapter describes materials and methods used routinely during the studies presented in Chapters Three and Four. Methods which apply specifically to work covered in individual chapters are detailed in the materials and methods section of the relevant chapter.

2.2 Materials

The following list comprises the consumables used for the DNA analysis presented in this thesis and their sources.

2.2.1 DNA

Oligonucleotide primers:		Cruachem Ltd, Glasgow, UK.
Markers:	λ-*Hind*III/*Eco*RI:	Appligene, Oncor, Watford, UK.
	100-bp DNA ladder:	Pharmacia Biotech Ltd, Milton Keynes, UK.
		Life Technologies Ltd, Paisley, UK.

2.2.2 Modifying Enzymes

Dynazyme:	Flowgen Instruments Ltd, Lichfield, UK.
Taq polymerase:	Pharmacia Biotech Ltd, Milton Keynes, UK.
Primezyme:	Biometra, Göttingen, Germany.

2.2.3 Restriction Enzyme

*Bam*HI restriction endonuclease: Promega Corporation, Southampton, UK.

*Nco*I restriction endonuclease: Promega Corporation, Southampton, UK.

*Taq*I restriction endonuclease: Promega Corporation, Southampton, UK.

2.2.4 Chemicals

Tris base, boric acid, ethidium bromide, orange G, xylene cyanol FF, bromophenol blue, sodium chloride, mineral oil, sodium perchlorate, ammonium persulphate, tetramethylethylenediamine (TEMED), Triton X-100, spermidine: Sigma Chemical Co Ltd, Poole, UK.

Formamide, agarose, acrylamide (19:1 with bis-acrylamide, 'Ultrapure'): Life Technologies Ltd, Paisley, UK.

Chloroform, ethanol, amberlite resin, bromophenol blue, sodium acetate, sucrose, magnesium chloride, sodium hydroxide, ethylene diamine tetraacetic acid (EDTA), sodium dodecyl sulphate (SDS): BDH Ltd, Poole, UK.

Urea: Appligene, Oncor, Watford, UK.

Sodium chloride: Fisher Scientific UK, Loughborough, UK.

Dynawax: Flowgen Instruments Ltd, Lichfield, UK.

Ultrapure dNTPs: Pharmacia Biotech Ltd, Milton Keynes, UK.

1.5 mM $MgCl_2$ optimised buffer for Dynazyme: Flowgen Instruments Ltd, Lichfield, UK.

1.5 mM $MgCl_2$ optimised buffer for *Taq* polymerase: Pharmacia Biotech Ltd, Milton Keynes, UK.

1.5 mM $MgCl_2$ optimised buffer for Primezyme: Biometra, Göttingen, Germany.

2.2.5 Radioisotope

α^{32} P-radiolabelled dCTP: Amersham International plc, Buckinghamshire, UK.

2.2.6 Miscellaneous Items

Polaroid film type 667: HA West, Clydebank, UK.

Kodak x-ray film: HA West, Clydebank, UK.

2.2.7 DNA Purification Systems

QIAamp® Blood Kit: QIAGEN Ltd, Crawley, UK

Nucleon II Kit: Scotlab Biosciences, Coatbridge, UK

Nucleon BACC 2 Kit: Scotlab Biosciences, Coatbridge, UK

2.3 Methods

2.3.1 Patient Selection

Unrelated patients referred to the periodontal department of Glasgow Dental Hospital for treatment were recruited for the study of genetic polymorphisms in GEOP. They were white and of European origin. The Glasgow Dental Hospital Ethics Committee approved the studies undertaken in this thesis and all the participants gave informed consent.

The clinical diagnosis of the patients was based on the following classification for GEOP (Hart *et al.*, 1991):

- the patient should be aged 35 years or less at diagnosis and should be systemically healthy

- interdental AL of $\geq$ 5 mm should be present on at least eight permanent teeth, at least three of which should not be first molars or incisors.

Clinical diagnoses were confirmed by evidence of interproximal BL on full mouth periapical radiographs taken using the parallel technique.

2.3.2 Control Groups

The control groups are distinctive to the particular studies and will be discussed in the relevant sections.

2.3.3 Blood Samples

10 ml of venous blood was collected from the ante cubital fossa in vials containing Potassium EDTA. The blood was stored in 50 ml polypropylene tubes at –70°C, until DNA separation could be carried out.

2.3.4 DNA Purification

Extraction of whole genomic DNA from nucleated peripheral blood cells was carried out using the Nucleon II protocol a and the more recently developed Nucleon BACC 2 protocol. These methods include the following steps: cell lysis; deproteinisation with sodium perchlorate; extraction with chloroform and the proprietary resin; and DNA precipitation. The extracted DNA was stored at 4°C until required. These protocols were performed according to the manufacturer's instructions as summarised below:

1. 40 ml of Reagent A was added to 10 ml of whole blood in a 50 ml polypropylene centrifuge tube. The tube was rotary-mixed for 4 min at room temperature and then centrifuged at 1300 x g for 4 min. The supernatant was discarded without disturbing the cell pellet.

2. 2 ml of Reagent B was added and the mixture was briefly vortexed to resuspend the cell pellet.

3. 500 µl of sodium perchlorate was added to the mixture and the tube was incubated in a shaking water bath at 65°C for 25 minutes.

4. 2 ml of chloroform (stored at 4°C and kept on ice) was added to the tube. The tube was rotary mixed for 10 min and then centrifuged at 800 x g for 1 min.

5. 300 µl of Nucleon silica suspension was added. The tube was centrifuged at 1300 x g for 3 min.

6. Only the DNA containing phase above the Nucleon layer was transferred into a fresh centrifuge tube of appropriate volume.

7. The tube was centrifuged briefly at 1300 x g to pellet any residual Nucleon silica and the supernatant carefully decanted to a fresh tube.

8. Two equal volumes of cold absolute ethanol at 4°C was added to the aqueous DNA containing phase. The tube was inverted gently to precipitate the DNA.

9. The DNA was pelleted, the supernatant decanted and the pellet air-dried.

Alterations to the above protocol for the Nucleon® BACC 2 DNA extraction kit were as follows. At stages 3 and 4 the contents of the tube were mixed by inverting at least 7 times by hand, rather than being incubated in a shaking water bath or rotary-mixed and centrifuged. After stage 8 the DNA precipitate was removed from the absolute alcohol and immersed in 70% ethanol.

2.3.5 Agarose Gel Electrophoresis

Agarose was dissolved in 1 x TBE buffer (Table 2.1) to a concentration of 0.8% w/v by heating in a microwave oven. Ethidium bromide (3 mg/ml) was added to the molten gel (15 µl to 100 ml 1 x TBE buffer). The cooled mixture was poured into a gel electrophoresis tank, the well-forming comb inserted and the gel allowed to set. Once the gel had solidified, the comb was removed carefully. The gel slab was submerged in 1 x TBE buffer. Sample DNA was mixed with 0.1 volume of gel loading dye (Table 2.1) before loading into the wells. A constant voltage of 70V was applied to the gel until the dye front had migrated to the bottom of the gel. DNA was visualised on a UV TM-40 transilluminator (UVP inc, USA) at 302 nm and photographed using Polaroid type 667 film.

Table 2.1 General stock solutions and buffers.

10 x TBE

Tris base	108 g
Boric acid	55 g
0.5M EDTA [pH 8.0]	40 ml
dH_2O	to 1 litre

Use at a final concentration of 1 x. Sterilise by autoclaving.

Reagent A

10mM Tris-HCl	1.2 g
320mM Sucrose	109.5 g
5mM $MgCl_2$	1 g
1% Triton X-100	10 ml
Adjust to pH 8.0 using 40% NaOH	
dH_2O	to 1 litre

Autoclave prior to use.

Reagent B

400mM Tris-HCl [pH to 8.0 using 40% NaOH]	48.4 g
60mM EDTA	22.3 g
150mM NaCl	126.6 g
1% SDS	10 g
(add SDS after autoclaving)	
dH_2O	to 1 litre

Ethidium Bromide (3 mg/ml)

Ethidium bromide	30 mg
dH_20	to 10 ml

Store away from light. Use at a final concentration of 0.5 μg/ml.

TE buffer (pH 8.0)

10mM Tris pH 8.0

1mM EDTA pH 8.0

0.5M EDTA (pH 8.0)

EDTA	186.1 g
dH_2O	900 ml

Adjust to pH 8.0 with NaOH. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving.

1M Tris-HCl (pH 8.0)

Tris base	121 g
dH_2O	900 ml

Adjust to pH 8.0 with HCl. Make up volume to 1 litre with dH_2O. Sterilise by autoclaving.

1% SDS

Sodium dodecyl sulphate	1 g
dH_2O	900 ml

Dissolve on heated stirer. Adjust to pH 7.2 with HCl. Make up volume to 1 litre with dH_2O.

Bromophenol blue loading dye

Bromophenol blue	0.05% w/v
Glycerol	50%

Orange G loading dye

Orange G	0.05% w/v
Glycerol	50%

Chapter 3 DNA Analysis of *HLADQB1* Polymorphism in Generalised Early Onset Periodontitis

3.1 Introduction

Immune response genes are found on various chromosomes and although their site of expression may be different they all have a similar phenotypic outcome i.e. they determine ability to respond to antigenic stimuli (McDevitt and Chinitz, 1969; Ríhová, 1995). Some investigators accept that *HLA* genes are immune response genes rather than markers linked to disease susceptibility genes found nearby on chromosome six (Hill, 1992).

Most of the polymorphisms found in the *HLA* complex are associated with the amino acids, which make up the antigen binding sites (Bjorkman *et al.*, 1987; Brown *et al.*, 1988; 1993; Stern *et al.*, 1994). Therefore, theoretically different polymorphisms should determine the binding of different antigens. Furthermore, certain infectious diseases have been associated with a particular HLA type. Hill *et al.* (1991) found a protective effect of HLA-Bw53 against both severe malarial anaemia and cerebral malaria in African children. In addition, on examination of class II haplotypes composed of HLA-DR and -DQ antigens they also found another protective association with HLA-DRw13.02. However, the latter was only statistically significant for individuals suffering from severe malarial anaemia.

It appears possible that variations in frequency of *HLA* allelic polymorphisms may determine susceptibility to EOP through their ability to bind immunodominant antigens of periodontal pathogens. The primary immune response to a 65 kDa protein of Aa has been demonstrated to be controlled by genes in the *IA* subregion of the *H2* complex (*MHC*) in inbred strains of mice (Nitta and Ishikawa, 1993). It is also possible that class I and II *HLA* alleles are markers rather than mutant genes (Riley and Olerup, 1992). In certain diseases mutant alleles in the *TNF* cluster have been shown to be in linkage disequilibrium with alleles spanning the whole of the *HLA* complex (Pociot *et al.*, 1993; Monos *et al.*, 1995). Considering the importance of TNF in the

in the pathogenesis of periodontitis, a mutant gene in the TNF complex might show linkage disequilibrium with a marker allele in the *HLADQ* or *DR* regions in EOP. Other genes involved in processing and transport of HLA antigens may also be linked to the class II gene cluster.

Over the past 20 years, researchers have periodically reported associations between the different forms of periodontitis and HLA antigens (Terasaki *et al.*, 1975; Kaslick *et al.*, 1975; Kaslick, West and Chasens, 1980; Saxén and Koskimies, 1984; Goteiner and Goldman, 1984; Cogen *et al.*, 1986; Klouda *et al.*, 1986; Katz *et al.*, 1987; Amer *et al.*, 1988; Shapira *et al.*, 1994b). Until recently, no convincing association between HLA type and any category of periodontitis had been demonstrated. These studies investigated polymorphisms defined serologically using the complement-dependent microlymphocytotoxicity test assay of the HLA antigens displayed on the cell surface (Terasaki and Singal, 1969; Terasaki *et al.*, 1978). The disadvantage of this technique was that it relied on alloantibody and monoclonal antibody reactive with specific antigens and was therefore difficult to standardise between laboratories. In addition, reagents were not available for all antigens. It was also not possible to distinguish between a "blank" antigen and a homozygous antigen without typing an entire family (Wade, 1996).

Despite the drawbacks of this technique, three independent research groups have reported similar findings. A study in Israel found a significant association between HLA-DR4 and GEOP (Katz *et al.*, 1987), but the patient sample size was small ($n = 10$). Alley *et al.* (1993) supported this finding in a Caucasian population. They investigated HLA-D phenotypes in individuals with or without IDDM and moderate to severe periodontitis. They observed a significantly elevated frequency of HLA-DR4 in diabetics and periodontitis patients. The association was stronger in periodontitis ($p = 0.001$) than diabetes ($p = 0.05$) and followed a similar pattern in HLA-DR53 and HLA-DQ3 which are in linkage disequilibrium with HLA-DR4. Firatli *et al.* (1996b) more recently reported a significant association between LEOP and GEOP and HLA-DR4 in a Turkish population.

In order to investigate *HLA* allelic polymorphisms in more detail molecular genetic techniques can be employed. A recent study in France confirmed the association between HLA-DR4 and GEOP. Sequence-specific oligoprobe hybridisation after PCR (PCR.SSO) was used and a more specific association, with *DRB1*0401*, *0404*, *0405* and *0408* alleles, was found for the –DR4 subgroup (Bonfil *et al.*, 1999). Another study in a population of Japanese patients found no association between class II HLA antigens and EOP using the complement-dependent microcytotoxicity method. However, a significant association was found between an atypical *Bam*HI restriction site in the *HLADQB1* gene and EOP (Takashiba *et al.*, 1994; Ohyama *et al.*, 1996) (Figure 3.1). This site was identified, following RFLP analysis, with cDNA probes for the *HLADRB1* and *HLADQB1* genes. The genes were digested into fragments of various lengths by the restriction enzymes *Bam*HI, *Eco*RI, *Msp*I and *Pst*I. The *HLADQB1* gene was split into a common fragment of 10.0 kb and uncommon fragments of 7.0 kb and 3.0 kb by *Bam*HI (Figure 3.1). The atypical site was concluded to be in the intron downstream of the third exon, after examination of fragments resulting from digestion of the 10.0 kb and 7.0 kb fragments with *Eco*RI and *Sma*I.

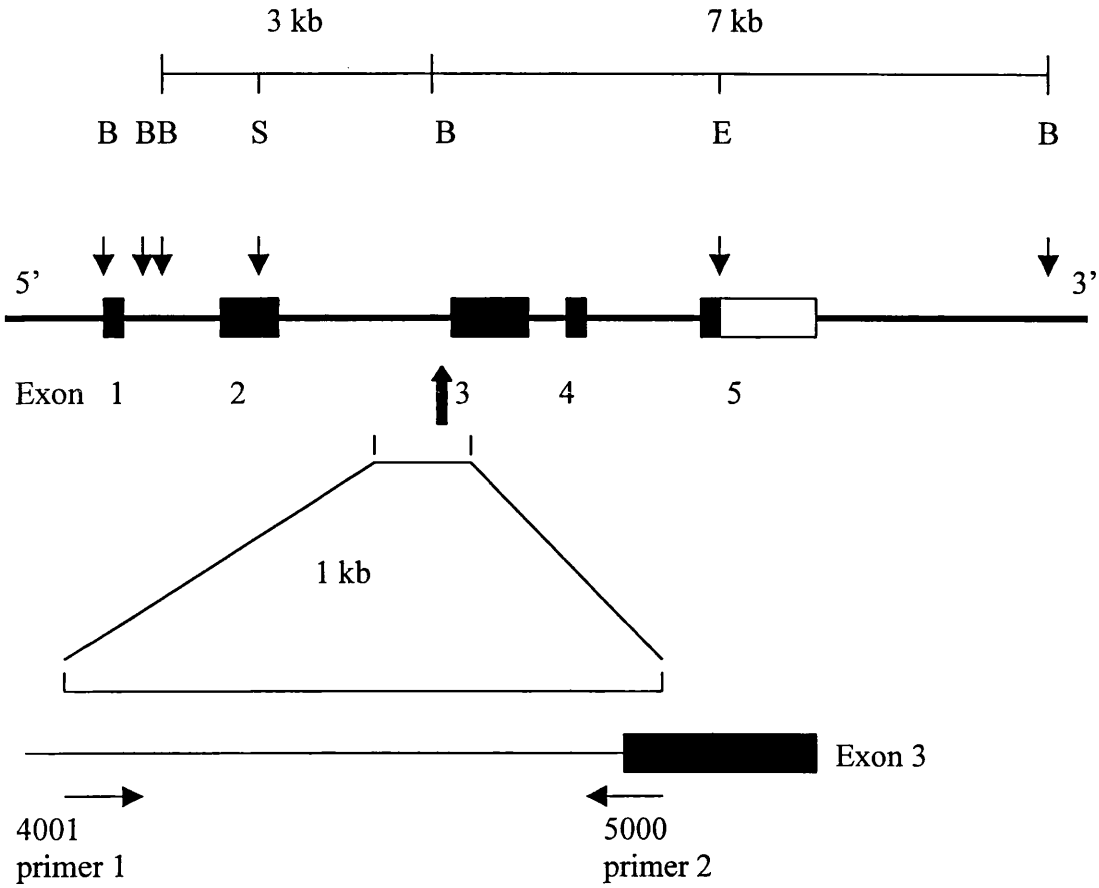
In the study presented here, the atypical *Bam*HI site was investigated in a population of European Caucasian GEOP patients.

3.2 Materials and Methods

3.2.1 Patient Selection

Forty-five patients were recruited to this study based on the criteria described previously (section 2.3.1). There were six patients recruited to the study who were over the age of 35 years when they presented in the periodontal department of Glasgow Dental Hospital and School. However, these patients had a clear history of having symptoms of the disease prior to the age of 35 years. In addition they were referred to the department by their general dental practioners for treatment of severe

Figure 3.1 Diagrammatic representation of the human *HLADQB1* gene.



Adapted from Jonsson *et al.* (1987), Larhammar *et al.* (1983) and Takakshiba *et al.* (1994). The one kilobase region which was amplified by PCR is magnified. The atypical *Bam*HI site is illustrated by a blocked arrow. The oligonucleotide primers correspond to the sequence reported by Larhammar *et al.* (1983). B = *Bam*HI, E = *Eco*RI, S = *Sma*I restriction sites.

Translated exon Untranslated exon Intron

progressive periodontitis. They had BL $\geq$ 50% around most of the affected teeth.

3.2.2 Control Group

The control group included 44 volunteers from the staff of Glasgow Dental Hospital with no evidence of GEOP. They were all white and of European origin. It was not considered to be important to match the controls for age. However, no individual under the age of 21 was recruited because 20 years was thought to be the lower limit at which signs of GEOP would be evident (Albandar *et al.*, 1997).

Demographic data for the patient and control groups are presented in Table 3.1.

3.2.3 DNA Purification

DNA separation from the EOP patients' blood was carried out using the Nucleon II protocol a and the more recently developed Nucleon BACC 2 protocol as previously described (section 2.3.4).

Extraction of whole genomic DNA from the control bloods was accomplished using the QIAamp ® Blood Kit. This is a rapid method of DNA extraction. It involves one step cell lysis, deproteinisation and extraction. The DNA is then precipitated, washed and eluted. Extracted DNA was stored at 4°C until required. This protocol was performed according to the manufacturer's instructions with some minor modifications as follows:

1. 200 µl of sample was pipetted into a 1.5 ml microfuge tube.

2. 25 µl QIAGEN Protease stock solution and 200 µl of Buffer AL were added and mixed immediately by vortexing.

3. The mixture was incubated at 70°C for 10 min.

4. 210 µl of 100% ethanol was added to the tube and the contents were mixed again by vortexing.

Table 3.1 Demographic data.

	Total[a]	Male[a]	Female[a]	Smokers[a]	Non-smokers[a]	Mean age[b]
Patients	45 (100)	19 (42)	26 (58)	23 (51)	22 (49)	31.36 (16-38)
Controls	44 (100)	18 (41)	26 (59)	12 (27)	32 (73)	33.07 (22-53)

[a] Numbers of individuals in each group; percentages in parentheses.

[b] Mean age and range in parentheses, in years.

5. A QIAamp spin column was placed into a 2 ml collection microtube (provided). The mixture from step 4 was carefully added to the QIAamp spin column without moistening the rim. The cap was closed and the tube was centrifuged at 8000 rpm (6000 x g) for 1 min. The QIAamp spin column was placed into a clean 2 ml collection microtube (provided) and the tube containing the filtrate was discarded.

6. The QIAamp spin column was carefully opened and 500 µl of Buffer AW was added. The tube was centrifuged at 8000 rpm (6000 x g) for 1 min. The filtrate was discarded and the QIAamp spin column was replaced into the same collection microtube.

7. The QIAamp spin column was carefully opened and another 500 µl of Buffer AW was added. The tube was centrifuged at full speed for 3 min.

8. The QIAamp spin column was placed into a clean 1.5ml microcentrifuge tube (not provided) and the collection microtube containing the filtrate discarded.

9. The QIAamp spin column was carefully opened and the DNA was eluted with 200 µl of Buffer AE (provided) preheated to 70°C.

10. The tube was incubated at 70°C for 5 min and centrifuged at 8000 rpm (6000 x g) for 1 min.

11. The DNA was then ready for further processing as required.

DNA integrity was checked and DNA quantitated using agarose gel electrophoresis (section 2.3.5). One microlitre of sample was added to 8 µl of molecular biology grade water (MBG) and 1 µl of bromophenol blue loading dye before loading into the gel.

3.2.4 Screening of Subjects for the Atypical *Bam*HI Restriction Site

3.2.4.1 PCR

In order to screen subjects for the atypical *Bam*HI site, PCR amplification of a 1.0 kb region corresponding to nucleotide residues 4001-5000 of the *HLADQB1* gene, was carried out (Larhammar *et al.*, 1983) (Figure 3.1).

The following synthetic *HLADQB1* specific oligonucleotide primers were used (Takashiba *et al.*, 1994):

primer 1: 5'-GAGTGCCTTTTAATTGGGGTG-3'

primer 2: 5'-GTAGACGTCTCCACGCTGGGG-3'

A master mix containing all the reagents minus the sample DNA was set up on ice as shown in Table 3.2. Primers were supplied as lyophilised pellets. They were resuspended in MBG H_2O at a concentration of 1 μg/μl and aliquoted. Hot start PCR, where the primers are separated from other components of the PCR by 50 μl of prewarmed wax (Dynawax), was used in this study. The wax prevents the reaction from starting until melting of the wax occurs on commencement of thermal cycling. The hot start method improves the specificity and yield of PCR products. One microlitre of sample DNA was added to the upper layer. Negative PCR controls contained 1 μl of MBG water instead of sample DNA. An Omnigene thermal cycler (Hybaid Ltd, Teddington, UK.) was employed for the PCR reactions. An initial denaturation step at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 1 minute, annealing of primers at 60°C for 2 minutes and primer extension at 72°C for 2 minutes. A final extension step at 72°C for 10 minutes completed the cycling. Ten microlitres of each PCR product were mixed with 1 μl of bromophenol blue loading dye and electrophoresed on a 2% agarose gel as described in section 2.3.5, with a 100-bp DNA ladder being used as a size marker.

Table 3.2 Upper and lower master mixes used in 'hot start' PCR.

1. Upper master mix

Reaction component	Concentration in final volume	Vol per 100 µl (per reaction)
Primer 1	0.2 µM	0.2 µl
Primer 2	0.2 µM	0.2 µl
Template DNA	–	1.0 µl
MBG H_2O	–	48.6 µl

2. Lower master mix

Reaction Component	Concentration in final volume	Vol per 100 µl (per reaction)
PCR Buffer (10 x)	1 x	10.0 µl
Dynazyme/*Taq* polymerase	2 units	1.0 µl
dNTPs (20mM)	0.2 mM	1.0 µl
MBG H_2O	–	38.0 µl

3.2.4.2 Purification of PCR product

The volume of PCR product was made up to 100 µl. DNA was precipitated by the addition of one-tenth volume (10 µl) of 3M sodium acetate and two volumes of ethanol (220 µl), followed by mixing and storage at –70°C for 30 min. The mixture was then centrifuged for 10 min at 13,000 rpm in a microcentrifuge to precipitate the DNA pellet. The supernatant was carefully removed and the DNA pellet air dried for 10 min and finally the DNA product was resuspended in 15 µl of MBG water.

3.2.4.3 Restriction endonuclease digestion of the PCR product

Amplified DNA was digested with *Bam*HI in a volume of 20 µl. Six microlitres of sample DNA were added to the digestion mixture which comprised 2 µl of recommended 10 x restriction enzyme buffer (supplied by the enzyme manufacturer), 0.5 µl *Bam*HI (10 units/µl), 0.4 µl 2mM spermidine and MBG water to 20 µl. Digestion reactions were thoroughly vortex-mixed and incubated at 37°C for a minimum of three hours. The digested samples were subjected to electrophoresis on 2.0 % agarose gels as described previously (section 2.3.5).

3.2.4.4 Contamination controls

Separate rooms were used for PCR and post-PCR analysis and a one-way system operated from the PCR laboratory. Laboratory coats were changed on entering and leaving the different rooms. These measures were imposed to prevent contamination of the PCR laboratory with extraneous products.

Equipment and reagents used in PCR were separated into batches and aliquoted for storage to prevent contamination of stock solutions. Aliquoting of reagents and all PCR experiments were carried out in a UV-irradiated laminar flow cabinet. All equipment was irradiated before use for 10 min with 1200 µJ min^{-1} at 254 nm in a UV crosslinker. All reagents were handled using filter tips to prevent aerosol contamination of the inside of the pipette. Gloves were changed whenever the operator's hands re-entered the flow cabinet having left to use the microcentrifuge or collect reagents. Negative controls were included with every PCR experiment to

check for contamination. Template DNA was added using positive displacement tips in a Template Tamer cabinet (Oncor, Appligene, Watford, UK).

3.2.5 Statistical Analysis

Statistical analysis of the data was carried out using the chi-squared test. This test compares the two sets of data in a 2 x 2 table and returns a probability value of the difference between them. The level of significance for this test is set at 0.05.

Table 3.3 Stock solutions and buffers.

QIAGEN Protease

Lyophilized QIAGEN protease	25 mg
MBG H_2O	1.4 ml

Store at – 20°C in 200 µl aliquots.

Buffer AL

Reagent AL 1	1 part
Reagent AL 2	4 parts

Store at room temperature in 5 ml aliquots.

N.B. Do not add QIAGEN Protease directly to Buffer AL.

Buffer AW

Buffer AW concentrate	17 ml
Ethanol (96-100%)	40 ml

Store at room temperature in 10 ml aliquots.

20 mM dNTPs (2' deoxynucleotide 5' triphosphates)

dATP (100mM)	200 µl
dTTP (100mM)	200 µl
dGTP (100mM)	200 µl
dCTP (100mM)	200 µl
MBG H_2O	to 1 ml

Store at –20°C in 50 µl aliquots.

10 x Optimised PCR Buffer

10mM Tris-HCl [pH 8.8 at 25°C]

1.5mM $MgCl_2$

50mM KCl

0.1% Triton X-100

Store at –20°C in 200 µl aliquots.

3M NaOAc

NaOAc	273.4 g
dH_2O	900 ml

Adjust to pH 5.5 with HCl. Make up to 1 litre with dH_2O. Sterilise by autoclaving.

Spermidine (100mM)

Spermidine	145.2 mg
MBG H_2O	10 ml

Use at 2mM final concentration.

3.3 Results

3.3.1 Purified Genomic DNA

DNA integrity was tested by running genomic DNA samples and marker fragments on 0.8 % agarose gels to check for shearing (Figure 3.2). Sheared samples would have appeared as a smear on the gel. The marker used in this example was λ-*Hind*III which is visible as bands of 23.5-, 9.4-, 6.7-, 4.3-, 2.3-, 2.0- and 0.6-kb on the gel.

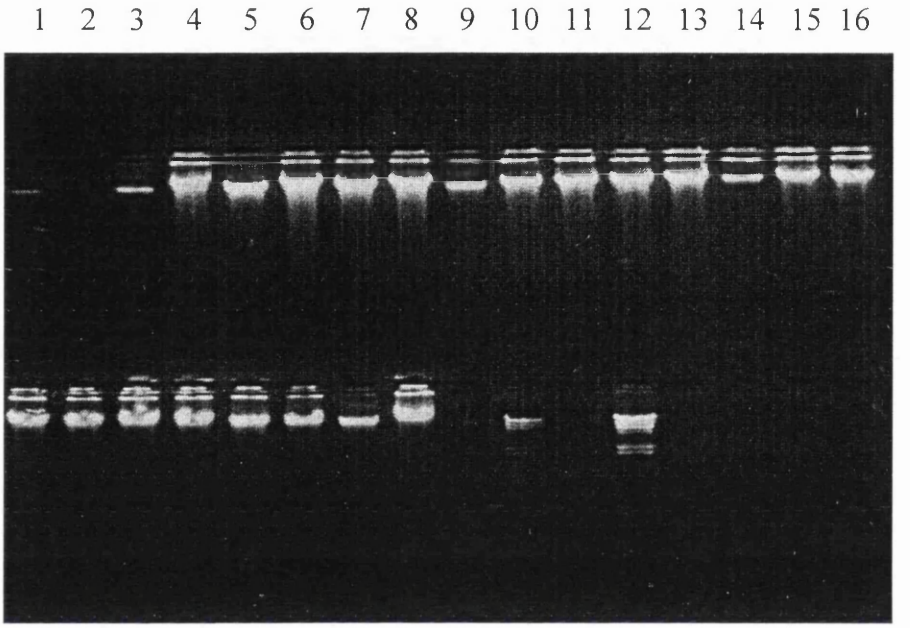
3.3.2 PCR Products

To confirm amplification of the 1.0 kb product samples were run on 2.0 % agarose gels, an example of which can be seen in Figure 3.3. A 100-bp marker was used to verify the size of the products.

3.3.3 PCR Digests

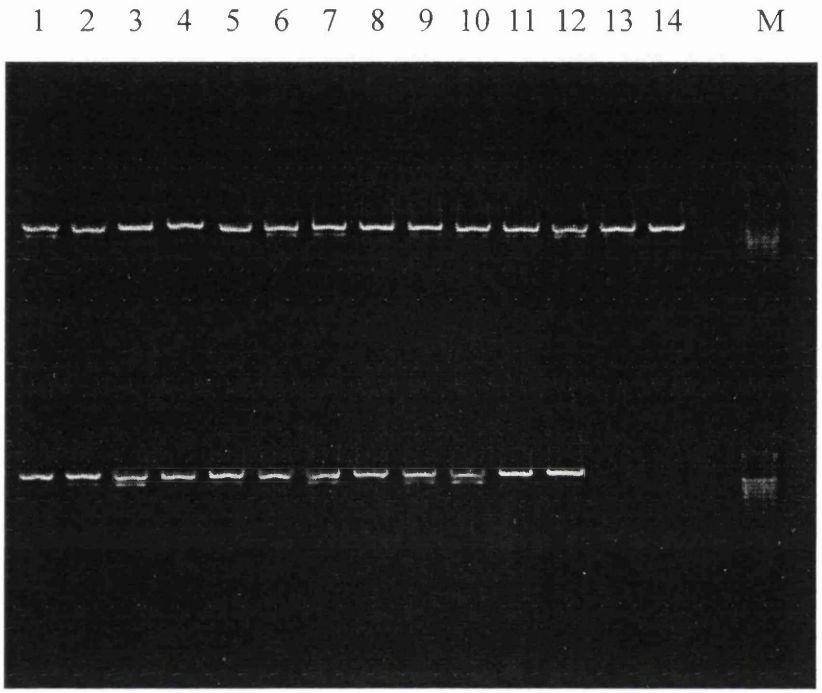
In order to investigate the presence or absence of the atypical restriction site, digests were run on 2.0 % agarose gels. Figure 3.4 shows a photograph of a typical gel. Fragments of 1000 bp (allele 2) and approximately 700 bp and 300 bp (allele 1) are seen for patients and controls with the atypical *Bam*HI restriction site, which demonstrates heterozygosity for the site. The size of the digested fragments differed from those reported by the Japanese researchers. They found intact fragments of 1000 bp but digested fragments of 650 and 350 bp. This may be due to differences in size estimation from the gels. It is also possible that the restriction site is in a slightly different position in Caucasians. The presence of an intact 1000 bp fragment indicates lack of the atypical *Bam*HI site. None of the patients or controls was homozygous for allele 1. Twenty-three patients (51%) and 23 controls (52%) showed evidence of the restriction site (Table 3.4). Data for smokers is shown separately from non-smokers. No statistically significant differences were found between patients and controls ($p = 0.91$). When smoking status was included in the analysis as a covariate there was still no significant difference

Figure 3.2 0.8% Agarose gel showing purified genomic DNA.



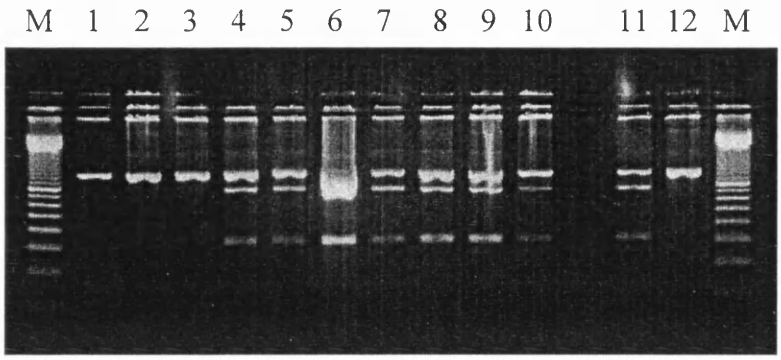
Lanes 1 and 3-24 demonstrate successful DNA purification. M = *λHind*III marker.

Figure 3.3 2.0% Agarose gel showing PCR products.



PCR products of 1000 bp can be seen in lanes 1-26. Lane 27 was left empty and lane 28 was the negative control. M = 100 bp marker.

Figure 3.4 2% Agarose gel showing digested PCR products.



Lanes 1, 2, 3 and 12 show undigested 1000 bp product (homozygous for allele 2). Lanes 4-11 show undigested 1000 bp product and DNA fragments of approximately 300 bp and 700 bp (heterozygous). M = 100 bp marker. The 800bp fragment shows up more strongly than the other marker fragments.

Table 3.4 Distribution of *HLADQB1* alleles in GEOP patients and controls.

Allele	Patients n (%)			Controls n (%)		
	S	NS	T	S	NS	T
1/2	12 (52)	11 (50)	23 (51)	6 (50)	17 (52)	23 (52)
2/2	11 (48)	11 (50)	22 (49)	6 (50)	15 (47)	21 (48)

S = smokers, NS = non-smokers, T = total, n = number in each group.

between the patient and control groups (smokers - p = 0.90, non-smokers – p = 0.82).

3.4 Discussion

3.4.1 Natural Selection

The results of this study do not support the finding of an association between an atypical *Bam*HI restriction site in the *HLADQB1* gene and GEOP in a population of Caucasian patients of European origin. It has been shown that variations in *HLA* allelic frequencies exist between different races and populations (Riley and Olerup, 1992). These variations may be reflected in associations between certain diseases and HLA type within different races (Baines and Ebringer, 1992; Awata and Kanazawa, 1994; Firatli *et al.*, 1996b). An explanation of this phenomenon is provided by studies of human evolution. The evolution of the *MHC* differs from that of most other single copy genes. Whereas mutations of single copy genes are driven by chance (genetic drift) those in the *MHC* are dominated by natural selection, specifically positive selection (Erlich and Gyllensten, 1991; Klein *et al.*, 1993; Hughes and Hughes, 1995).

Elucidation of the structure of HLA molecules provides evidence for the theory of natural selection. In addition, the serotype HLA-Bw53 which has a protective effect against malaria is common only among sub-Saharan Africans where it is found at levels of up to 40% (Hill *et al.*, 1991). The protective class II antigen (HLA-DRw13.02) is also found more frequently in African populations. It thus seems likely that geographical variation in exposure to *Plasmodium falciparum* has contributed to the development of protective *HLA* polymorphisms.

There appear to be differences in the prevalence of certain bacteria in subgingival plaque in patients from different countries and ethnic groups. One study in a Chilean population showed a high prevalence and high levels of Pg and Pi at diseased sites, contrary to many previous North American and European studies of LEOP (Lopez, Mellado and Leighton. 1996). However, Aa was only found in about one-third of patients and one-quarter of diseased sites. Another study in a group of Chinese patients with LEOP found no

evidence of Aa in the sites they examined. The predominant species cultured included Gram positive rods (Naiming *et al.*, 1991). Schenkein *et al.* (1993) found that Pg was more significantly associated with black AP patients and Fn was associated with white AP- and EOP-affected individuals.

Crossover of pathogenic species between the different diagnostic categories of periodontitis and periodontally healthy individuals has also been recorded. These phenomena may be partly due to researchers investigating evidence of a bacterial species rather than attempting to isolate a particular strain or serotype in a population. For example, specific genetic variants of Aa were found to correlate with disease and health in LEOP families from West Philadelphia (DiRienzo *et al.*, 1994). Haubek *et al.* (1995) also provided evidence for the absence in North European Caucasians of especially virulent clones of Aa in patients with AP and LEOP. In contrast, a highly virulent clone of Aa producing increased quantities of leucotoxin has been isolated from a number of LEOP patients, from families of African origin living in geographically widespread locations (Haubek *et al.*, 1996). More recently another group found a significant association between the detection of a high-leucotoxin-producing strain of Aa and the development of disease in children from African American families with LEOP (Bueno, Mayer and DiRienzo, 1998). Gunsolley *et al.* (1991) found that higher levels of antibody titres to Aa serotypes b and c were found in black GEOP patients and non-periodontitis subjects than in whites. This relationship between IgG levels and race appeared to be restricted to Aa. In another study, Fn subspecies *nucleatum* showed a strong association with AP; however, Fn subspecies *polymorphum* was isolated mainly from healthy sites (Gharbia *et al.*, 1990).

These investigations suggest that differences in *HLA* genotype between racial groups may influence susceptibility to infectious periodontal diseases. They may explain the lack of association between the atypical *Bam*HI site in the *HLADQB1* gene and GEOP in Caucasian patients reported in this study.

3.4.2 Linkage Disequilibrium

In addition to linkage between the *TNF* complex and class II *MHC* alleles, strong linkage disequilibrium has been demonstrated between the *DR* and *DQ* subregions of *HLA* class II genes (Inoko *et al.*, 1986; Jonsson *et al.*, 1987; Pociot *et al.*, 1993). A disease-associated allele at one of the *DR* loci could be detected by linkage to a polymorphism in the *DQ* subregion. This is interesting considering the finding of an association between HLA-DR4 and severe periodontitis in three racially distinct populations, using HLA serotyping techniques (Katz *et al.*, 1987; Alley *et al.*, 1993; Firatli *et al.*, 1996b). Furthermore, a very recent study confirmed this association using molecular genetic techniques (Bonfil *et al.*, 1999). The sequencing of the promoter regions of class II loci has also revealed marked polymorphisms for *DRA*, *DRB*, *DQA* and *DQB*. The latter show strong linkage disequilibrium with the corresponding *HLA* class II alleles (Schwartz, Felser and Mayr, 1995).

Recently a number of genes have been identified within the *HLA* complex which code for proteins involved in processing and transport of class I and II HLA glycoproteins. For example, *TAP* (transporter associated with antigen processing) genes located within the *HLA* class II gene cluster code for transporter proteins (Momburg, Neefjes and Hämmerling, 1994; Ríhová, 1995). These proteins are involved in transport of antigenic peptides from the cytosol into the endoplasmic reticulum where they are combined with class I antigens, which will eventually be presented at the cell surface to cytotoxic T cells. *TAP* genes also show polymorphism, which may be responsible for disease susceptibility (Powis *et al.*, 1993; Aoki, Isselbacher and Pillai, 1993). This would be dependent on which pathogenic peptides are allowed to gain access to the endoplasmic reticulum and eventually be presented to T cells (Ríhová, 1995). Strong linkage disequilibrium has been found between *TAP2* and *HLADR*, *DQ* and *DO* (Schwartz, Felser and Mayr, 1995). In other words an association between an *HLADQ* allele and a particular disease could be indicative of a defect in class I peptide uptake into the endoplasmic reticulum.

Other genes found in the class II cluster code for proteins, which are important for antigenic loading of class II peptides. These are known as HLA-DM molecules

(Pieters, 1997). The *HLADM* genes are much less polymorphic than classical *HLA* class II genes (Bodmer, 1995). However, class II loading is defective in cells not expressing these genes (Mellins *et al.*, 1990; Denzin *et al.*, 1994).

This discussion highlights the many genes within the *HLA* complex which may be responsible for causing susceptibility to infection. If *HLA* class II genes are markers for immune response genes found nearby on chromosome six, the marker allele might vary between different populations and racial groups (Baines and Ebringer, 1992). This would provide an explanation for the findings of the study reported here.

3.4.3 Cell-Surface Trimming of Class II MHC Bound Peptides

The importance of intracellular processing in MHC antigen presentation has been well documented (reviewed by Pieters (1997)). In addition, aminopeptidase-N (CD13) mediated cell-surface trimming of MHC class II bound peptides has been demonstrated in mice lymphoma cell lines (Larsen *et al.*, 1996). The aminopeptidase-N can digest the NH2-terminal end of the bound peptide modifying antigen recognition by the TCR. The authors suggested that the surface of the APC could alter the MHC class II bound peptides if intracellular processing yields products which are too long to fit within the binding groove. They also hypothesised that peptide carboxy-terminals protruding from the groove might be digested by a carboxypeptidase, with equally important effects on the sensitivity and specificity of the T-helper cell response. The complexity of the pathways involved in antigen processing and presentation, and in T cell recognition, allows the reader to understand the difficulty of associating a particular *HLA* allele with EOP.

3.4.4 Periodontitis Is a Mixed Infection

In the Japanese study (Takashiba *et al.*, 1994), the atypical *HLADQB* gene variation was found in 10 of 30 EOP patients, one of 24 AP patients and three of 42 healthy controls. The authors suggested that the atypical site might be useful as an indicator for a subpopulation of EOP patients and may affect immune reactions such as antigen recognition.

Periodontitis is believed to be a mixed infection caused by a small group of specific Gram negative bacteria. It is possible that different subgroups of EOP patients are susceptible in the first instance to different species of bacteria and that this is reflected in their serum antibody response to these bacteria (Mooney *et al.*, 1995). These varying responses could be associated with different *HLA* genotypes within a homogeneous population. Hart and co-workers (Hart *et al.*, 1995; Hart, 1996) investigated the association of HLA-D allotypes with antibody response and periodontal disease category. Seventy-six black subjects (39 EOP, 22 AP and 15 controls) were typed for specific HLA class II antigens (DR4, D53, DQ3). These antigens had previously been found to be associated with periodontitis in a group of early-onset diabetics (Alley *et al.*, 1993). No association between any specific HLA type and any disease category was found. However, when 19 individuals were analysed according to IgG2 antibody titre to Aa and the presence of a specific antigen, DR53 was found to be associated with the ability to mount a high antibody titre.

Separation of the GEOP population investigated here into groups defined by, for example, antibody titre, cytokine levels or disease severity might have revealed an association with the *HLADQB* gene.

3.4.5 Immunodominant Antigens

One of the major problems of associating a particular *HLA* genotype with periodontal disease is the uncertainty that still exists regarding which species or strains of bacteria are pathogenic for particular disease categories (Schenkein, 1998). Evidence of associations of individual species and mixed bacterial infections with different diagnostic categories of periodontal disease has been reported (Socransky and Haffajee, 1997). However, which of the bacteria identified in microbiological studies are pathogenic and which are merely opportunists is still not clear. In addition, there is also uncertainty regarding which are the immunodominant antigens of these bacteria. The following suggestions have been made; an Mab 1A1 epitope common to multiple gene products of Pg (Curtis *et al.*, 1996); the fimbrial proteins of Pg and Aa (Evans *et al.*, 1992; Harano, Yamanaka and Okuda, 1995; Sojar, Hamada and Genco, 1997); lipopolysaccharide of Gram negative

bacteria (Vasel *et al.*, 1996); and heat shock protein 60 (HSP60) of Pg and Aa (Maeda *et al.*, 1994; Nakano *et al.*, 1995).

It should be emphasised that there is marked homology between HSP60 of Aa and several such proteins of other species of Gram negative bacteria. Nakano *et al.* (1995) found that rabbit polyclonal antibodies to the 64 kDa protein cross-reacted with the HSP60 of *Haemophilus aphrophilus*, *Haemophilus influenzae*, *Haemophilus paraphrophilus*, *Escherichia coli* and Ec. The HSP60 of Aa also cross-reacted with other bacteria and human HSP60. It is possible that human antibody responses to the Aa protein are in fact cross-reactive with HSP60 of other bacteria or self-antigens. Of interest was the lack of any cross-reactivity between the 64 kDa protein of Aa and any cellular proteins of Pg, Pi and Fn, other suspected periodontal pathogens. Cross-reactivity has also been demonstrated between LPS of Pg and Bf (Vasel *et al.*, 1996). Furthermore, investigators looking at the effects of LPS from non-oral bacteria have found cross-reactivity of an epitope in the Lipid A portion of the LPS molecule in a number of Gram negative bacteria (Seifert *et al.*, 1996). This immunodominant epitope would not be recognised by HLA class II molecules, which only present peptides.

The problem identified here is that there are many possible antigens implicated in periodontal disease. There must therefore be thousands of possible epitopes and combinations of epitopes on these antigens, and any of these may be important in antigen peptide presentation by HLA molecules. Until the microbial aetiology of periodontal disease is better understood and patient populations better defined, with regard to infecting microorganisms and immunodominant epitopes, it will be difficult to identify associations between the *HLA* complex and periodontitis.

3.4.6 Superantigens

An individual peptide, which forms a complex with an HLA molecule, will stimulate a specific clone of T cells. Recently new peptide molecules have been identified known as superantigens. These molecules are capable of activating T cells non-specifically without being presented in the peptide-binding groove of the HLA-D molecule (Zadeh and Kreutzer, 1996) (Figure 1.2). Superantigens interact directly with the Vβ region of

the T cell receptor and can therefore stimulate a much larger proportion of the T cell pool. *Staphylococcus aureus* enterotoxins have been identified as being superantigens. It is also possible that some periodontal pathogens produce superantigens (Mathur *et al.*, 1995; Zadeh and Kreutzer, 1996; Getka *et al.*, 1996). If this is the case, *HLA* alleles may not play an important role in determining susceptibility to periodontitis, which may explain the lack of an association between GEOP and the *HLADQB1* gene in this study.

3.4.7 *HLA* Genes May Modify the Immune Response in Periodontitis

In a subsequent study by the Japanese group (Ohyama *et al.*, 1996), DNA typing of 24 EOP patients and 47 controls was performed. The results of this study were inconclusive. A significantly increased frequency of *HLADQB1* (**0503* and **0602*) alleles was found in EOP patients. However, the atypical *Bam*HI site was only found in relation to *DQB1*0602* and not *DQB1*0503*. The site was also detected in all but one individual who carried alleles *DQB1*0604* and *DQB1*0605*, which were not found to be associated with EOP (Ohyama *et al.*, 1996). An additional level of polymorphism has been demonstrated by PCR amplification and sequencing of alleles detected by RFLP. A single RFLP may be associated with more than one sequence (Riley and Olerup, 1992). The authors (Ohyama *et al.*, 1996) stated in their discussion that the lack of strong linkage between *HLADQ* alleles and EOP is probably due to the multifactorial aetiology of the disease. No reference was made to the influence of smoking on susceptibility to EOP. In the present study no differences were observed between the presence of the atypical *Bam*HI site in smokers and non-smokers. It is possible that the genes of the *HLA* complex are not major susceptibility genes for EOP. They may instead have a modifying effect (Marazita *et al.*, 1996; Hart, 1996). This would explain the difficulty in finding convincing associations between *HLA* alleles and EOP, particularly in studies of small numbers of patients.

3.4.8 Concluding Remarks

The lack of an association between the atypical *Bam*HI site and GEOP in a European Caucasian population could reflect racial genetic variation in *HLA* allelic frequencies which could be due to natural selection. Variation in exposure to periodontal

pathogens between different racial groups and linkage disequilibrium between EOP and different alleles of the *HLA* complex are other explanations. *HLA* genes are probably not major susceptibility genes for EOP but may instead have a modifying effect. If this is the case, interactions could occur between the modifying gene and environmental factors, which vary between the populations studied.

Chapter 4 DNA Analysis of *IL10* and *TNF* Polymorphisms in Generalised Early Onset Periodontitis

4.1 Introduction

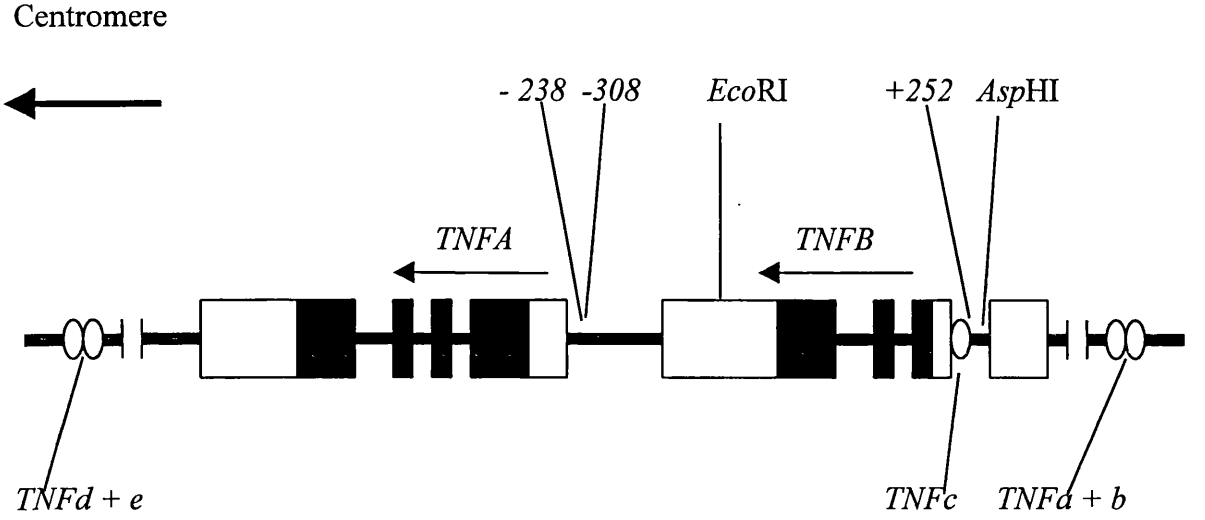
Cytokines are regulatory proteins of low molecular weight, which are active in very small amounts (femtomolar to picomolar concentrations). They transmit information between cells and form a complex network of interactions. They may act synergistically or may inhibit the action of another cytokine. Most cytokines are pleotropic. They are produced by activated cells and mainly exert their effects locally by binding to high affinity receptors on the surface of immune cells. The proinflammatory cytokines, IL-1 and TNFα, play a central role in the pathogenesis of many autoimmune and infectious diseases including periodontitis. Control of the production and function of these inflammatory proteins is accomplished by soluble receptors, high-affinity autoantibodies and anti-inflammatory cytokines such as IL-1ra and IL-10 (Bendtzen, 1994; Symons, Young and Duff, 1995). Interleukin-1 receptor antagonist regulates IL-1 function by binding to the same high affinity receptors as IL-1 without activating the cell. In contrast IL-10 suppresses cytokine synthesis both at the mRNA and protein levels (De Waal Malefyt *et al.*, 1991; De Waal Malefyt, Yssel and de Vries, 1993). Transcription of mRNA can be influenced by the binding of regulatory proteins to the promoter or enhancer regions of a particular gene (Connor and Ferguson-Smith, 1997; Knolle *et al.*, 1998). Gene activity may also be regulated later in the process of protein synthesis for example at the mRNA splicing stage (Howells, 1995). Alternatively the rate of translation may be altered.

Stable interindividual differences in cytokine secretion patterns have been reported (Mølvig *et al.*, 1988). It has also been demonstrated that allelic variation at cytokine loci, and in genes regulating their expression, may affect cytokine responses between individuals (Pociot *et al.*, 1991; 1993; Messer *et al.*, 1991; Derkx *et al.*, 1995; Eskdale *et al.*, 1998a). In addition, associations between polymorphisms of candidate cytokine genes and certain diseases have been documented (Pociot *et al.*, 1991; 1993; McGuire *et al.*, 1994; Wilson *et al.*, 1994; Eskdale *et al.*, 1997).

Microsatellite loci are marker motifs consisting of non-coding dinucleotide repeats (Jacob, Mykytyn and Tashman, 1993). The number of repeats rather than the nucleotide sequence differentiates the allelic polymorphism. Although microsatellites have no known function, they are useful as markers of cytokine gene expression. Some microsatellites lie close to cytokine genes and are in linkage disequilibrium with them and their promotor and enhancer region.

The *TNF* gene cluster is 12 kb in length and lies within the *HLA* gene complex (Figure 1.1). It contains five polymorphic microsatellites (Figure 4.1) (Jongeneel *et al.*, 1991; Nedospasov *et al.*, 1991; Udalova *et al.*, 1993). In addition, several bi-allelic RFLP polymorphisms have been described; some of these are illustrated in Figure 4.1 (Partanen and Koskimies, 1988; Messer *et al.*, 1991; Wilson *et al.*, 1992; Ferencik *et al.*, 1992; D'Alfonso and Momigliano Richiardi, 1994). Polymorphic alleles within the *TNF* region have been found to correlate with TNFα and TNFβ production (Pociot *et al.*, 1991; 1993; Messer *et al.*, 1991; Braun *et al.*, 1996; Kroeger and Abraham, 1996; Wilson *et al.*, 1997). An increase in TNFα levels has been suggested to be due to up-regulation of the *TNFA* gene. Regulation of LPS induced secretion of IL-1 and TNFα has also been associated with particular HLA-DR subtypes (Mølvig *et al.*, 1988; Santamaria *et al.*, 1989; Jacob *et al.*, 1990). These findings suggest that class II *HLA* genes are in linkage disequilibrium with the *TNF* locus (Jongeneel *et al.*, 1991; Pociot *et al.*, 1993; Wilson *et al.*, 1993; Plevy *et al.*, 1996). However, it has also been shown that *TNF* alleles can influence levels of TNFα and the outcome of infection independently of the *HLA* complex (Mølvig *et al.*, 1990; McGuire *et al.*, 1994; Galbraith *et al.*, 1998). Gallagher *et al.* (1997) screened a large cohort from the West of Scotland for *TNF* polymorphisms and MHC serotypes. They found that the alleles at the *TNF* locus were in some cases an integral part of the entire *HLA* complex, in others alleles at particular loci varied between different ethnic populations. It was suggested that the identification of *TNF* alleles as disease markers independent of the *MHC* may require matching of patient and control groups at both class I and II loci.

Figure 4.1 **Diagrammatic representation of the human *TNFA* and *TNFB* (*LTA*) genes.**



The position of the five microsatellite loci *TNFa – TNFe* (Udalova *et al.*, 1993) are illustrated together with the following RFLP biallelic polymorphisms: *TNFA –238* (*Nla*IV) (D'Alfonso and Momigliano Richiardi, 1994); *TNFA –308* (*Nco*I) (Wilson *et al.*, 1992); *Eco*RI (Partanen and Koskimies, 1988); *TNFB +252* (*Nco*I) (Messer *et al.*, 1991); and *Asp*HI (Ferencik *et al.*, 1992).

Microsatellite — Intron — Exon

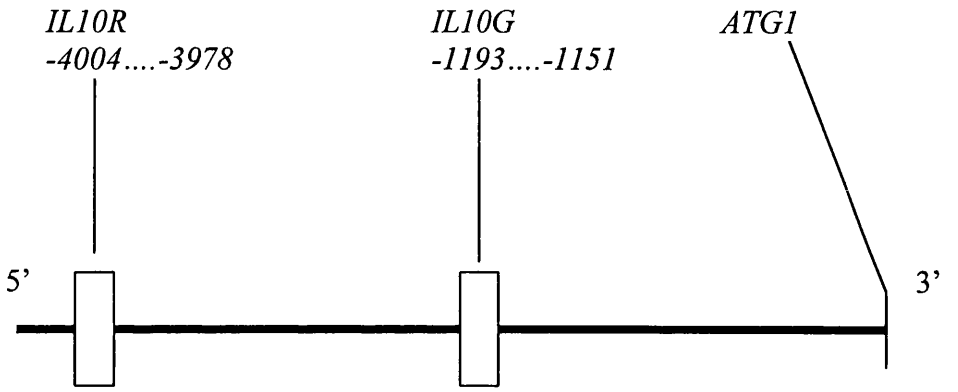
Untranslated exon — Interruption

Adapted from Wilson *et al.* (1995) and Rink and Kirchner (1996).

Recently Galbraith *et al.* (1998) demonstrated a significant association between increased TNFα production by oral PMN in AP patients and the heterozygous *T1,2* genotype of *TNFA -308* ($p = 0.037$) (Figure 4.1). When disease severity was also taken into account a significant association between the *T1,2* genotype and increased production of TNFα was only seen in patients with severe disease. These findings need further corroboration, as the numbers of individuals in the different categories of disease severity were small ($n = 10$ for mild, 10 for moderate, 12 for severe). In addition, only three patients in the severe group showed increased production of TNFα and were also heterozygous for the *TNFA -308* gene polymorphism. Because of the small size of the groups, no analysis of the contribution of smoking as a confounding variable could be carried out. Despite these drawbacks the results of this study are interesting, since a large-scale family linkage analysis of over a hundred families with EOP had previously indicated linkage for the *HLA* region of chromosome six (Wang *et al.*, 1996; Hart and Kornman, 1997). Many similarities exist in the pathogenesis of AP and EOP and the importance of TNF in mediating the destructive process has been highlighted (Roberts *et al.*, 1997). It seems possible therefore that TNF production in patients with EOP may be influenced by allelic polymorphisms in the *TNF* gene cluster.

Two microsatellites found within the promoter region of *IL10* have recently been elucidated (Figure 4.2) (Eskdale and Gallagher, 1995; Eskdale, Kube and Gallagher, 1996). Eskdale *et al.* (1998a) examined the occurrence of polymorphic alleles at these loci and IL-10 secretion levels. They found *IL10R3* was associated with lower production of IL-10 more often than any other allele at the same locus. In addition, the haplotype *IL10R2/IL10G14* was associated with highest IL-10 secretion and *IL10R3/IL10G7* was associated with lowest IL-10 secretion. The same group reported a higher frequency of *IL10R2* and a lower frequency of *IL10R3* in three geographically and racially diverse groups of RA patients compared with controls (Eskdale *et al.*, 1998b). Since patients with RA demonstrate higher levels of IL-10 in serum and synovial fluid it was suggested that IL-10 production may be under direct genetic control. An association was also found between the combined genotype (reduced *IL10G9* and increased *IL10G13*) and systemic lupus erythematosus

Figure 4.2 Diagrammatic representation of the human *IL10* promoter region.



Illustrated are the *IL10R* and *IL10G* microsatellites. *ATG1* represents the start of the *IL10* gene. ▯ Microsatellite ▬ Intron

Adapted from Eskdale *et al.* (1997).

(Eskdale *et al.*, 1997). Recently a role for IL-10 in the pathogenesis of periodontal disease was proposed (Stein and Hendrix, 1996; Stein *et al.*, 1997). It is possible that polymorphic alleles in the *IL10* microsatellites influence susceptibility to EOP.

This study presents data from the analysis of polymorphisms at *TNF* and *IL10* microsatellite loci in a population of GEOP patients compared with a control population. These microsatellite loci consist of dinucleotide repeats. The microsatellites examined were *TNFa*, comprising 14 alleles, and *TNFd* with seven alleles (Figure 4.1). The two microsatellites found within the *IL10* promoter region were also examined (Figure 4.2). *IL10R* (5 alleles) and *IL10G* (16 alleles) lie approximately 4 kb and 1.2 kb respectively from the *IL10* transcription initiation site (Eskdale and Gallagher, 1995; Eskdale, Kube and Gallagher, 1996).

4.2 Materials and Methods

4.2.1 Patient Selection

Seventy-five unrelated GEOP patients were recruited for the study of *TNF* and *IL10* microsatellite loci, according to the criteria previously described (section 2.3.1). Demographic data are presented in Table 4.1. Thirteen patients in this group were ≥ 35 years of age when they presented in the periodontal department of Glasgow Dental Hospital and School. The reasons for their inclusion have already been described in section 3.2.1.

4.2.2 Selection of Controls

The control group comprised unrelated healthy blood donors whose samples were provided by the tissue-typing laboratory at Glasgow Royal Infirmary. This group did not include any individuals with autoimmune or malignant disease or relatives of individuals with these diseases. They were ethnically-matched with the patient group. No demographic data were available for the control population. In addition, their periodontal status was not determined. However, this was not considered to be

Table 4.1 Demographic data of GEOP patient population.

	Total[a]	Male[a]	Female[a]	Smokers[a]	Non-smokers[a]	Mean age[b]
Patients	75 (100)	23	52	35 (46.67)	40 (53.33)	31.84 (16-43)

[a] Numbers of individuals in each group; percentages in parentheses.

[b] Mean age and range in years, in parentheses.

important since the frequency of GEOP in the general population (0.3%) is low (Oliver, Brown and Löe, 1998).

The control population for the *TNFa*, *IL10R* and *IL10G* microsatellites was previously presented as follows: *TNFa* (n = 91) by Gallagher *et al.* (1997); *IL10R* (n = 94) by Eskdale *et al.* (1996) and; *IL10G* (n = 102) by Eskdale and Gallagher (1995).

4.2.3 DNA Purification

DNA was extracted from the patients' blood using the BACC2 Nucleon kit (section 2.3.4). The resulting DNA samples were stored at 4°C until required. DNA integrity was checked and DNA quantitated using agarose gel electrophoresis (section 2.3.5). Two microlitres of DNA were added to 6.2 µl of TE buffer and 4 µl of orange G loading dye before loading into the gel. A constant voltage of 60V was applied to the gel for 30 to 60 minutes.

4.2.4 PCR Reactions

The microsatellite PCR reactions each required a different pair of primers (Table 4.2) and thermocycling conditions (see below). The thermocycler used was a Biometra Uno Thermoblock (Biometra, Göttingen, Germany).

Equipment and reagents used in PCR were separated into batches, and aliquoted for storage to prevent contamination of stock solutions. All reagents were handled using filter tips to prevent contamination of the inside of the pipette with aerosol. Negative controls were included with every PCR experiment to check for contamination. Positive controls from previous studies were used in the PCR reactions.

Template DNA was added to the thin-walled tubes and overlaid with mineral oil. The operator then moved to another bench where the master mix was prepared and added to the tubes. The master mixes for the different PCR reactions are detailed in Table 4.3.

Table 4.2 Sequences and positions of primers used in PCR reactions.

Name	Region	Approximate Position	Sequence 5' – 3'
TNF.2	5' NCR	3.5 kb from *TNFB*	GCC.TCT.AGA.TTT.CAT.CCA. GCC.ACA
TNF.4	5' NCR	3.5 kb from *TNFB*	CCT.CTC.TCC.CCT.GCA.ACA. CAC.A
TNF.12	3' NCR	8–10 kb from *TNFA*	CAT.AGT.GGG.ACT.CTG. TCT.CCA.AAG
TNF.11	3' NCR	8-10 kb from *TNFA*	AGA.TCC.TTC.CCT.GTG. AGT.TCT.GCT
IL10.1	5' NCR	1.2 kb from *IL10*	GTC.CTT.CCC.CAG.GTA. GAG.CAA.CAC.TCC
IL10.2	5' NCR	1.2 kb from *IL10*	CTC.CCA.AAG.AAG.CCT. TAG.TAG.TGT.TG
IL10.3	5' NCR	4 kb from *IL10*	CCC.TCC.AAA.ATC.TAT.TTG. CAT.AAG
IL10.4	5' NCR	4 kb from *IL10*	CTC.CGC.CCA.GTA.AGT. TTC.ATC.AC

Table 4.3 PCR master mixes.

1. *TNFa* and *TNFd* master mixes

Reaction Component	Stock concentration	Concentration in final volume	Vol per 20 µl (per reaction)
Primers *TNF.2/TNF.12*	10 µM	1.0 µM	2.0 µl
Primers *TNF.4/TNF.11*	10 µM	1.0 µM	2.0 µl
α^{32}P.dCTP	–	–	0.02 µl
dCTP	2 mM	0.2 mM	2.0 µl
dATP, dTTP, dGTP	20 mM	2.0 mM	
PCR Buffer	10 x	1 x	2.0 µl
Primezyme	–	0.4 units	0.2 µl
Template DNA	–	–	1.0 µl
MBG H_2O	–	–	10.78 µl

Table 4.3 PCR master mixes (continued).

1. *IL10G* master mix

Reaction Component	Stock concentration	Concentration in final volume	Vol per 25 µl (per reaction)
Primer *IL10.1*	10 µM	1.0 µM	2.5 µl
Primer *IL10.2*	10 µM	1.0 µM	2.5 µl
α^{32}P.dCTP	–	–	0.02 µl
dCTP	2 mM	0.2 mM	2.5 µl
dATP, dTTP, dGTP	20 mM	2.0 mM	
PCR Buffer	10 x	1 x	2.5 µl
Primezyme	–	0.5 units	0.25 µl
Template DNA	–	–	2.0 µl
MBG H_2O	–	–	12.73 µl

2. *IL10R* master mix

Reaction Component	Stock concentration	Concentration in final volume	Vol per 20 µl (per reaction)
Primer *IL10.3*	10 µM	1.0 µM	2.0 µl
Primer *IL10.4*	10 µM	1.0 µM	2.0 µl
α^{32}P.dCTP	–	–	0.02 µl
dCTP	2 mM	0.2 mM	2.0 µl
dATP, dTTP, dGTP	20 mM	2.0 mM	
PCR Buffer	10 X	1 X	2.0 µl
Primezyme	–	0.5 units	0.25 µl
Template DNA	–	–	2.0 µl
MBG H_2O	–	–	9.73 µl

Following PCR, 8 µl of formamide loading buffer was added to each sample and the samples were stored at –20°C until required.

4.2.4.1 *TNFa* and *TNFd* PCR

The *TNFa* primers, *TNF.2* and *TNF.4* (Table 4.2) are adjacent to the *TNFa* $CA_{(n)}$ microsatellite repeat sequence. The *TNFd* primers, *TNF.12* and *TNF.11* (Table 4.2) are adjacent to the *TNFd* $CT_{(n)}$ microsatellite repeat sequence.

Thermocycling was carried out as follows; an initial denaturation step at 94°C for 5 minutes was followed by 5 cycles of denaturation at 94°C for 25 seconds, annealing of primers at 65°C for 30 seconds and primer extension at 74°C for 30 seconds. Thirty cycles of 94°C for 15 seconds, 60°C for 20 seconds and 74°C for 30 seconds and a final extension step at 72°C for 10 minutes completed the PCR.

4.2.4.2 *IL10G* PCR

The *IL10G* primers, *IL10.1* and *IL10.2* (Table 4.2) complement bases –1254 to –1228 and –1117 to –1042 from the transcription start site of the *IL10* gene. These are 35 bp and 10 bp, respectively, from the ends of the *IL10G* microsatellite $CA_{(n)}$ repeat sequence.

Thermocycling was carried out at 94 °C for 5 minutes followed by 29 cycles of 94 °C for 15 seconds, 65°C for 1 minute, 72 °C for 1 minute and 72 °C for 5 minutes.

4.2.4.3 *IL10R* PCR

The *IL10R* primers, *IL10.3* and *IL10.4* (Table 4.2) complement bases –4058 to –4035 and –3967 to –3943 from the transcription start site of the *IL10* gene. This is 1 bp and 41 bp from the ends of the *IL10R* microsatellite $CA_{(n)}$ repeat sequence.

Thermocycling was carried out at 94 °C for 5 minutes followed by 30 cycles of 94 °C for 15 seconds, 61°C for 15 seconds, 72 °C for 15 seconds and 72 °C for 5 minutes.

4.2.5 Denaturing Polyacrylamide Gel Electrophoresis

The amplified PCR products were run on high resolution gels in order to differentiate the alleles by size in relation to control DNA of known genotype rather than size markers.

The polyacrylamide gel mix contained 6% acrylamide deionised with Amberlite resin, and urea. For *TNFa* and *TNFd* gels the urea concentration was 7.65M, otherwise it was 7M. The total volume of each gel mix was 100 ml. A volume of 750 µl 10 % w/v ammonium persulphate and 50 µl TEMED was added to the gel to initiate polymerisation. The gel was left overnight to polymerise before use.

The electrophoresis buffer was 1 x TBE. PCR samples were denatured at 80 °C, and cooled immediately on ice to prevent renaturation, before loading onto the gel. The gels had been preheated and were run at 75W on Stratagene BaseAce sequencing rigs (Stratagene, Cambridge, UK) for three hours. Xylene cyanol loading dye added to the samples migrates with fragments of 110 bp on a 6% acrylamide gel. Therefore this gave a visual indication of when separation of products was nearing completion.

The gels were allowed to dry and then exposed to X-ray film.

4.2.6 Statistical Analysis

Statistical analysis of the *TNF* and *IL10* polymorphisms was performed using the CLUMP programme devised by Sham and Curtis (1995). This programme is designed for use in genetic case-control studies where multiple alleles are being considered and where the numbers observed for some alleles are small. The significance of differences in allele frequencies is assessed in a 2 x N table using Monte Carlo simulations. The number of times that a chi-squared value associated with the observed data is achieved by simulated data sets is the measure of significance. This programme is designed to maximise the chi-squared value by "clumping" the columns together into a new 2 x 2 table. The statistic produced is the T4 value, which is equivalent to the chi-squared value of the difference in frequencies of all the alleles clumped together. The use of the Monte Carlo method avoids the need for a

Bonferroni correction and the difficulty of assessing the significance of rarer alleles. One thousand simulations were performed for each set of microsatellite data. A p-value of < 0.05 was taken to be statistically significant.

Table 4.4 Reagents and buffers.

Formamide loading buffer

95% Formamide

20mM EDTA

0.05% Bromophenol blue

0.05% Xylene cyanol FF

10 x Optimised PCR Buffer

10mM Tris-HCl [pH 8.8 at 25°C]

1.5mM $MgCl_2$

50mM KCl

0.1% Triton X-100

Store at –20°C in 200 µl aliquots.

20 mM dNTPs (2' deoxynucleotide 5' triphosphates)

dATP (100mM)	200 µl
dTTP (100mM)	200 µl
dGTP (100mM)	200 µl
MBG H_2O	to 1 ml

Store at –20°C in 50 µl aliquots.

4.3 Results

The results of the analysis of *TNFa*, *TNFd*, *IL10G* and *IL10R* polymorphisms are displayed in Figures 4.3 to 4.10. The PCR for *TNFd* was unsuccessful for one patient who was a smoker; for *IL10G* it was unsuccessful for two smokers and two non-smokers and for *IL10R* for three smokers and two non-smokers.

The results of the statistical analysis were as follows:

TNFa comparison of EOP and control populations: T4 = 10.23

p = 0.14

TNFa comparison of EOP smokers and non-smokers: T4 = 4.58

p = 0.75

TNFd comparison of EOP and control populations: T4 = 1.87

p = 0.72

TNFd comparison of EOP smokers and non-smokers: T4 = 1.30

p = 0.80

IL10G comparison of EOP and control populations: T4 = 10.07

p = 0.09

IL10G comparison of EOP smokers and non-smokers: T4 = 7.71

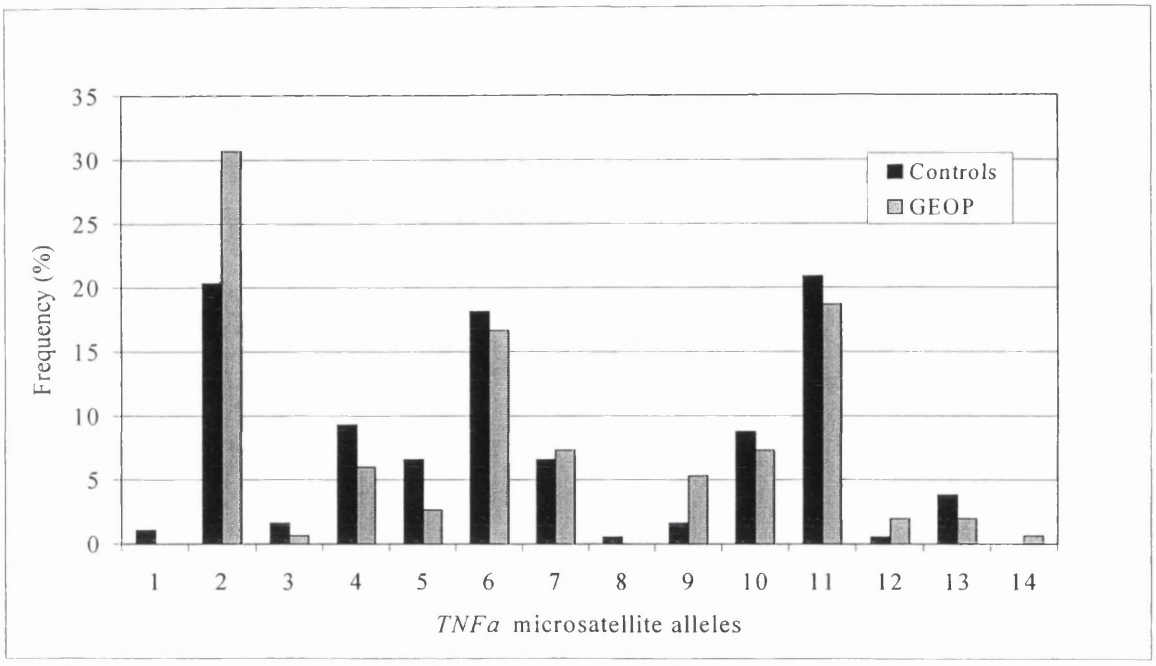
p = 0.16

IL10R comparison of EOP and control populations: T4 = 1.10

p = 0.57

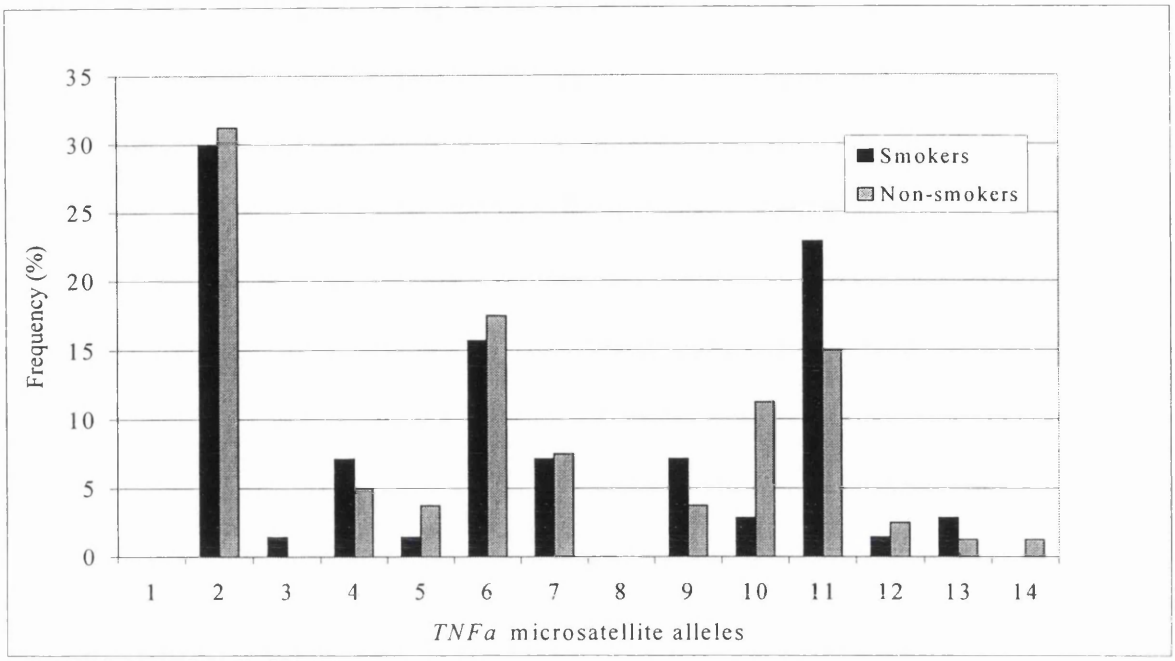
IL10R comparison of EOP smokers and non-smokers: T4 = 2.19

Figure 4.3 **Frequency of *TNFa* microsatellite alleles in GEOP and control populations.**



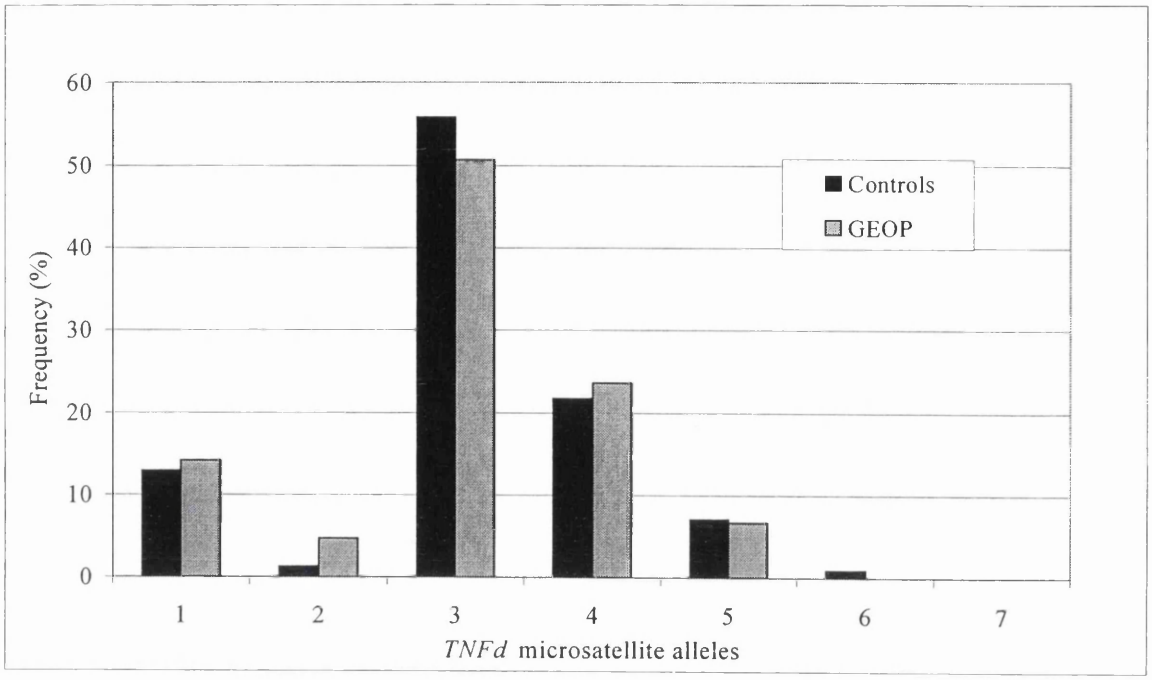
	Control Population (n = 91)		GEOP Population (n = 75)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	2	1.10	0	0.00
2	37	20.3	46	30.67
3	3	1.65	1	0.67
4	17	9.34	9	6.00
5	12	6.59	4	2.67
6	33	18.13	25	16.67
7	12	6.59	11	7.33
8	1	0.55	0	0.00
9	3	1.65	8	5.33
10	16	8.79	11	7.33
11	38	20.88	28	18.67
12	1	0.55	3	2.00
13	7	3.85	3	2.00
14	0	0.00	1	0.67
	182	100	150	100

Figure 4.4 **Frequency of *TNFa* microsatellite alleles in GEOP smokers and non-smokers.**



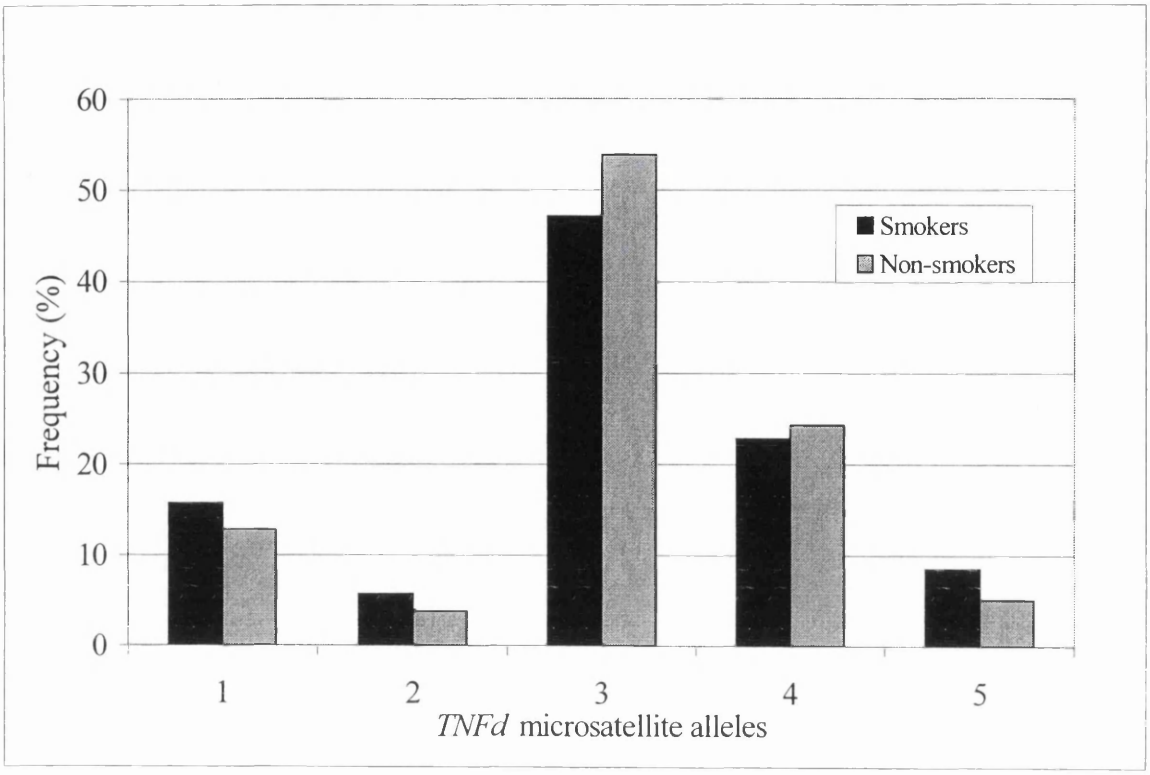
	GEOP Smokers (n = 35)		GEOP Non-smokers (n = 40)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	0	0.00	0	0.00
2	21	30.00	25	31.25
3	1	1.43	0	0.00
4	5	7.14	4	5.00
5	1	1.43	0	0.00
6	11	15.71	14	17.50
7	5	7.14	6	7.50
8	0	0.00	0	0.00
9	5	7.14	3	3.75
10	2	2.86	9	11.25
11	16	22.86	12	15.00
12	1	1.43	2	2.50
13	2	2.86	1	1.25
14	0	0.00	1	1.25
	70	100	80	100

Figure 4.5 **Frequency of *TNFd* microsatellite alleles in GEOP and control populations.**



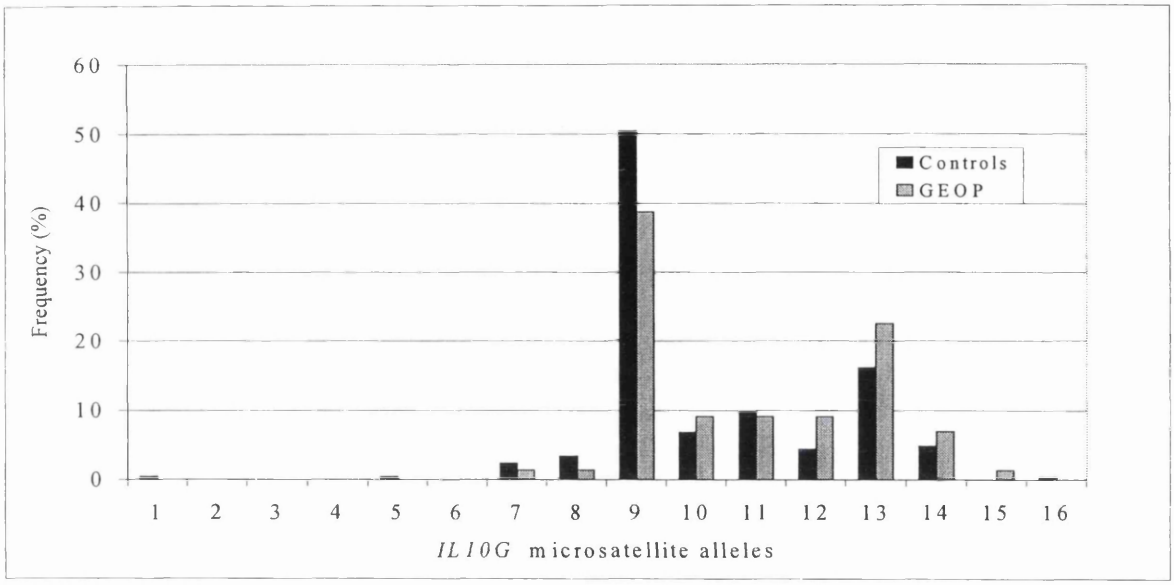
	Control Population (n = 154)		GEOP Population (n = 74)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	40	12.99	21	14.19
2	4	1.30	7	4.73
3	172	55.84	75	50.68
4	67	21.75	35	23.65
5	22	7.14	10	6.76
6	3	0.97	0	0.00
	308	100	148	100

Figure 4.6 **Frequency of *TNFd* microsatellite alleles in GEOP smokers and non-smokers.**



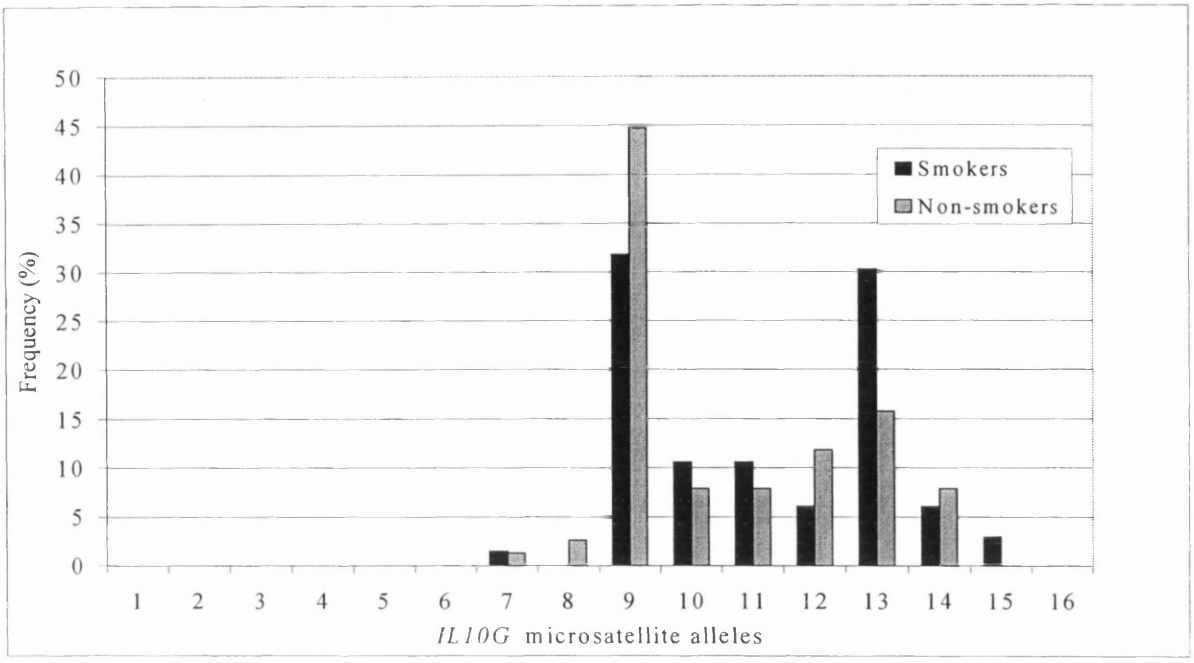
	GEOP Smokers (n = 35)		GEOP Non-smokers (n = 39)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	11	15.71	10	12.82
2	4	5.71	3	3.85
3	33	47.14	42	53.85
4	16	22.86	19	24.36
5	6	8.57	4	5.13
	70	100	78	100

Figure 4.7 **Frequency of *IL10G* microsatellite alleles in GEOP and control populations.**



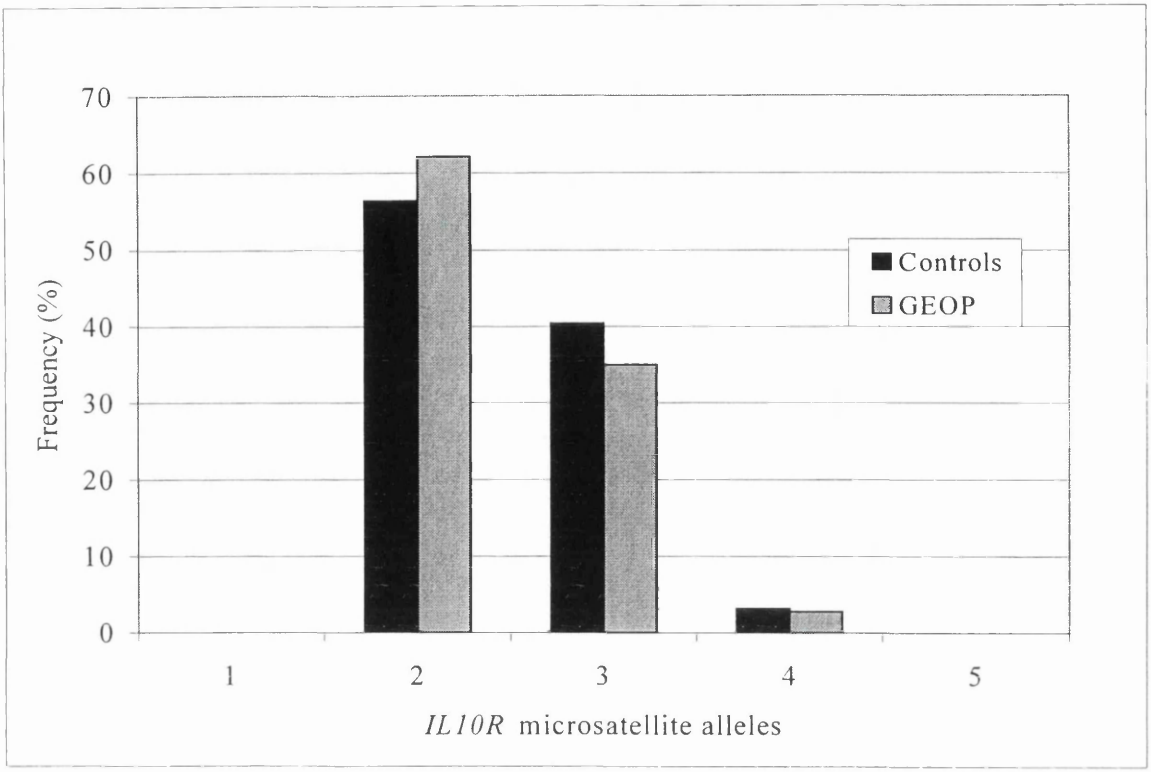
	Control Population (n = 102)		GEOP Population (n = 71)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	1	0.49	0	0.00
2	0	0.00	0	0.00
3	0	0.00	0	0.00
4	0	0.00	0	0.00
5	1	0.49	0	0.00
6	0	0.00	0	0.00
7	5	2.45	2	1.41
8	7	3.43	2	1.41
9	103	50.49	55	38.73
10	14	6.86	13	9.15
11	20	9.8	13	9.15
12	9	4.41	13	9.15
13	33	16.18	32	22.54
14	10	4.9	10	7.04
15	0	0.00	2	1.41
16	1	0.49	0	0.00
	204	100	142	100

Figure 4.8 **Frequency of *IL10G* microsatellite alleles in GEOP smokers and non-smokers.**



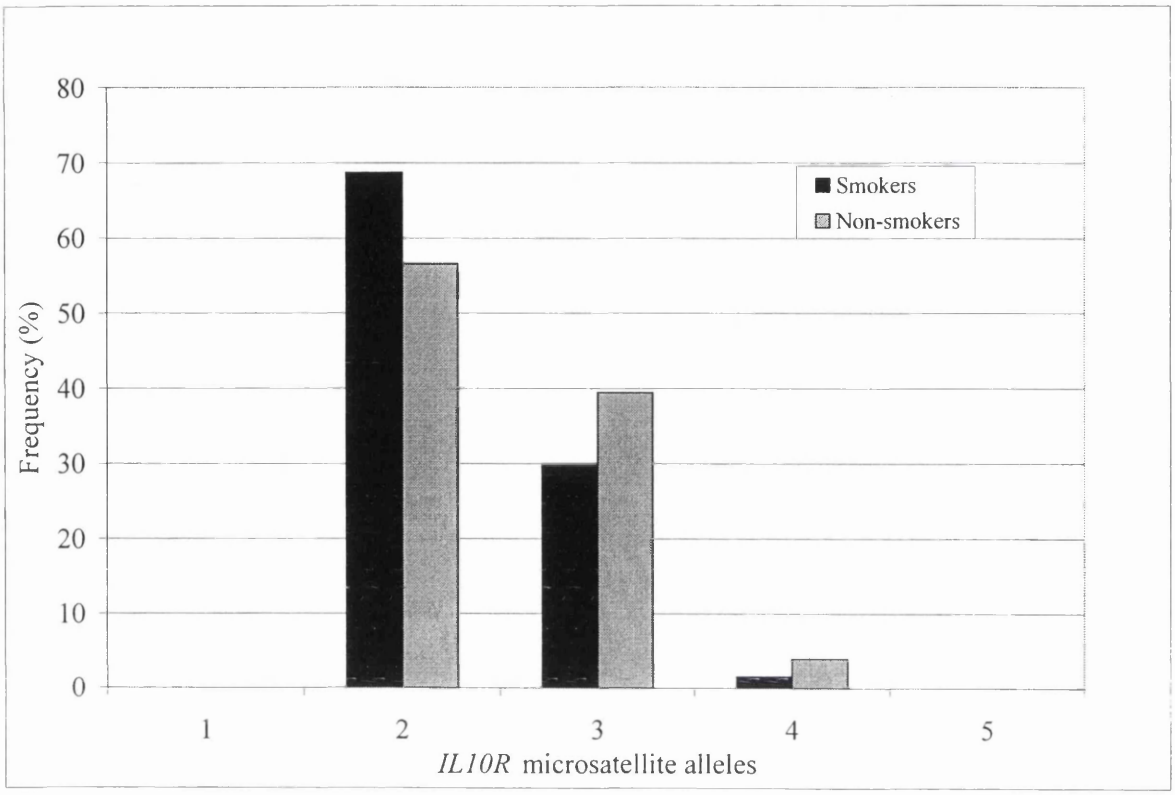
	GEOP Smokers (n = 33)		GEOP Non-smokers (n = 38)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
7	1	1.52	1	1.32
8	0	0.00	2	2.63
9	21	31.82	34	44.74
10	7	10.61	6	7.89
11	7	10.61	6	7.89
12	4	6.06	9	11.84
13	20	30.30	12	15.79
14	4	6.06	6	7.89
15	2	3.03	0	0.00
16	0	0.00	0	0.00
	66	100	76	100

Figure 4.9 **Frequency of *IL10R* microsatellite alleles in GEOP and control populations.**



	Control Population (n = 94)		GEOP Population (n = 70)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	0	0.00	0	0.00
2	106	56.40	87	62.14
3	76	40.40	49	35.00
4	6	3.20	4	2.86
5	0	0.00	0	0.00
	188	100	140	100

Figure 4.10 Frequency of *IL10R* microsatellite alleles in GEOP smokers and non-smokers.



	GEOP Smokers (n = 32)		GEOP Non-smokers (n = 38)	
Allele	Number Observed	Frequency (%)	Number Observed	Frequency (%)
1	0	0.00	0	0.00
2	44	68.75	43	56.58
3	19	29.69	30	39.47
4	1	1.56	3	3.95
5	0	0.00	0	0.00
	64	100	76	100

$p = 0.29$

No significant associations were found between any of the microsatellite loci individually and GEOP. In addition, there were no significant differences in frequency of any alleles between GEOP smokers and non-smokers. A trend towards a decrease in *IL10G9* and an increase in *G13* between GEOP patients and controls was noticed (Figure 4.7). When the patient group was split into smokers and non-smokers it became evident that this difference was due to the smokers (Figure 4.8). There was also a trend towards an increase in *TNFa2* in GEOP (Figure 4.3), although there were no differences in frequency between GEOP smokers and non-smokers for this microsatellite (Figure 4.4).

The trend in *TNFa2* in GEOP has been found to be significant in other studies of chronic inflammatory diseases, often as part of a haplotype which in one report of Crohn's disease also included an increased frequency of *TNFd4* (Pociot *et al.*, 1993; Plevy *et al.*, 1996; Field *et al.*, 1997). The data was therefore also analysed for frequency of the *TNFa2TNFd4* allelic combination.

The combined genotype was found to be more frequent in the patient group (33.8%) than in the control group (21.2%). This difference between the two populations was statistically significant ($p = 0.047$). There was however no difference in the combined genotype between GEOP smokers and non-smokers ($p = 0.82$).

4.4 Discussion

4.4.1 Advantages of Dinucleotide Repeats as Genetic Markers

A useful way of differentiating allelic polymorphisms at the DNA level is to employ the PCR-RFLP method. This is especially helpful in highly polymorphic areas of the genome, such as the *MHC*, where haplotypes extend across the entire region (Pociot *et al.*, 1991; Wilson *et al.*, 1993). In addition, linkage of certain diseases to various cytokine genes has been demonstrated using this method (Pociot *et al.*, 1992; McGuire *et al.*, 1994; McDowell *et al.*, 1995).

The use of microsatellite motifs as genetic markers has been developed more recently (Weber, 1990). This is a much more informative method of gene mapping. Microsatellite loci consist of variable lengths of tandem repeats of untranslated DNA, which are locus specific. The size of the tandem repeats has been shown to remain constant and it appears therefore that they are stable markers which are interspersed throughout the genome (Jacob, Mykytyn and Tashman, 1993). These short sequences are much more polymorphic than RFLP and are multiallelic. Normally only two alleles are identified using RFLP (Botstein *et al.*, 1980) which has a maximum polymorphism information content (PIC) value of 0.375. For dinucleotide repeats this value may be doubled (Weber, 1990; Jongeneel *et al.*, 1991).

Microsatellite loci lie in close proximity to immune response genes, such as cytokine genes and also their promoter and enhancer regions (Jacob, Mykytyn and Tashman, 1993; Danis *et al.*, 1995). Therefore they may play a part in regulating genes encoding protein products which have a role in the immune and inflammatory response. Only a small number of cytokine genes show polymorphism using restriction enzymes. Until the discovery of microsatellite loci a low degree of sequence polymorphism of the *TNF* locus had been demonstrated using RFLP (Udalova *et al.*, 1993). Typing of *TNF* microsatellites and one RFLP defined by the restriction enzyme *Nco*I (*TNF +252*) (Figure 4.1) has revealed at least 35 distinct haplotypes (Wilson, di Giovine and Duff, 1995). Since microsatellite loci are more informative as genetic markers than RFLP, their use in examining associations with heterogeneous diseases such as EOP may be more rewarding.

4.4.2 Analysis of the *TNF* Microsatellite Loci

No significant association was found between the *TNFa* locus or the *TNFd* locus and GEOP in the present study. There was however a trend towards an increase in allele 2 of *TNFa* in GEOP patients compared with controls (Figure 4.3). When the data for GEOP smokers and non-smokers was analysed there was no difference between the two groups (Figure 4.4). The trend towards an increase in *TNFa2* observed in GEOP has been found to reach significance in other chronic inflammatory diseases including RA and Crohn's disease (Plevy *et al.*, 1996; Hajeer *et al.*, 1996; Field *et al.*, 1997). In

these studies the association was found to be part of an extended haplotype including other *TNF* markers and also previously described *HLA* allelic combinations. These conditions share in common with periodontitis high levels of proinflammatory cytokines, such as IL-1 and TNFα, at sites of tissue destruction. Raised TNFα secretion has been found to be predictive of acute relapses, following steroid induced remission for one year, in Crohn's disease (Schreiber *et al.*, 1999). In addition, anti-TNFα monoclonal antibody administered to patients with both RA and Crohn's disease results in a dramatic improvement in clinical symptoms (Elliott *et al.*, 1994; van Dullemen *et al.*, 1995). Recently a highly significant association between sulcus bleeding index, probing depth and clinical AL, and longstanding active RA ($p = 0.0001$) was reported (Käßer *et al.*, 1997). The RA patient group and the control group were comparable for age, sex, smoking and oral hygiene status.

Insulin-dependent diabetes mellitus is another condition, which shows a strong association with *MHC* loci, namely *HLADR4* and *TNFa* (Davies *et al.*, 1994; Gustke *et al.*, 1998). Patients with IDDM have an increased susceptibility to periodontitis (Cianciola *et al.*, 1982; Firatli, 1997). Alley *et al.* (1993) found significant associations between HLA-DR4, -DR53 and -DQ3 and diabetes and periodontitis. The association with peridontitis was highly significant ($p = 0.001$) whereas the association with diabetes was moderately significant ($p = 0.05$). These findings were recently confirmed in another study, which examined antibody responses to cell antibody-proteins of *Capnocytophaga* species (Dyer *et al.*, 1997). Serum IgG responses and HLA-DR4 were found to be significantly correlated with diabetes and periodontitis. The authors suggested that both IDDM and periodontitis patients have a reduced antibody response to Capnocytophaga, which may predispose to infection with this bacteria. Furthermore, they proposed that the HLA-D type and altered immune function might play a more important role in susceptibility to periodontitis in IDDM than diabetes itself. Three other groups have also reported a significant association between HLA-DR4 and EOP (Katz *et al.*, 1987; Firatli *et al.*, 1996b; Bonfil *et al.*, 1999). The studies by Katz *et al.* (1987) and Bonfil *et al.* (1999) were in small groups of patients ($n = 10$ and $n = 11$ respectively). However, the latter group typed patients and controls using PCR.SSO and found a significant association with the *HLADR4* subtypes *DRB1*0401*, **0404*, **0405* and **0408*. This group of alleles had previously

been implicated in RA (Bonfil *et al.*, 1999). Pociot *et al.* (1993) found an increased frequency of the *TNFa2* microsatellite allele in IDDM. This was part of an extended haplotype including HLA-DR4 and allele two of the *TNFB +252* RFLP (Figure 4.1). Individuals carrying this haplotype had an increased TNFα secretory capacity.

Recently an association between an increased frequency of the combined *TNFa2TNFd4* genotype and Crohn's disease was reported (Plevy *et al.*, 1996). Neither allele was associated individually with Crohn's disease. The finding in this study of a significant association between the *TNFa2TNFd4* allelic combination and GEOP is therefore interesting in the light of the foregoing discussion. However, the association is only marginally significant and requires further investigation and corroboration in cases and controls also typed for HLA class I and II antigens.

Taken together, the results of these studies raise the possibility of an underlying disease genotype, or a few genotypes, which predispose to a number of chronic inflammatory conditions. These genotypes might contribute to defects in one or more aspects of the immune response, which have a generalised effect. Susceptibility to disease could be governed by interaction between the disease genotype and a mutant gene for a particular body system. Alternatively, environmental factors such as smoking or infection acting both locally and systemically might cause manifestation of a disorder in a host carrying a defective immune response genotype.

4.4.3 Analysis of the *IL10* Microsatellite Loci

No significant associations were found between the *IL10* microsatellite loci and GEOP in this study. However, a trend towards an association between polymorphisms at the *IL10G* microsatellite and EOP was seen. Figure 4.7 demonstrates the distribution of alleles for this locus between the EOP and control populations. Allele nine is decreased and allele 13 increased in EOP patients compared with controls. A recent paper by Eskdale *et al.* (1997) found a similar association between systemic lupus erythematosus (SLE) and this locus. It was suggested that this finding might relate to the increased levels of IL-10 seen in patients with SLE (Llorente *et al.*, 1994). IL-10 is a potent stimulator of B cell proliferation and differentiation (Rousset *et al.*, 1992; Itoh

and Hirohata, 1995) and can prolong B cell survival by preventing apoptosis (Levy and Brouet, 1994). It has been shown that IL-10 stimulates the production of autoantibodies in SLE (Llorente *et al.*, 1995). In addition, an increasing IL-10:IFNγ–secreting-cell ratio with increasing disease severity has been demonstrated in SLE, indicating a Th-2 type of response. This finding is of interest since a number of investigators have found a predominantly Th-2 response in periodontitis lesions (Wassenaar *et al.*, 1995; Tokoro *et al.*, 1997). It has recently been demonstrated that increased production of IL-10 by GMC from AP lesions and from peripheral blood monocytes of diabetics stimulated with Pg occurs (Stein and Hendrix, 1996; Stein *et al.*, 1997). It was suggested that autoimmune reactions against gingival tissues may be triggered by IL-10 (Stein *et al.*, 1997).

A subsequent study by Eskdale *et al.* (1998a) found the haplotype *IL10R2/IL10G14* was associated with highest IL-10 secretion and *IL10R3/IL10G7* was associated with lowest IL-10 secretion. This group also demonstrated a significant association between RA and a high frequency of *IL10R2* at the expense of *IL10R3* in three geographically diverse patient populations (Eskdale *et al.*, 1998b). Serum and synovial fluid collected from RA patients contain significantly elevated levels of IL-10 (Cush *et al.*, 1995). It was suggested that the increased production of IL-10 may contribute to the reduced T cell response and increased antibody and rheumatoid factor titres in RA patients. These findings indicate that the decreased frequency of *IL10G9* and increased frequency of *IL10G13* in SLE, and the trend in GEOP, may not have a direct relationship with IL-10 levels. Another group found significant associations between *IL10G* and *bcl2* microsatellite loci and SLE in Mexican Americans (Mehrian *et al.*, 1998). The *bcl2* gene codes for a molecule that inhibits or delays apoptosis, (Levy and Brouet, 1994) and is found on chromosome 18. Susceptibility alleles of *IL10* and *bcl2* were found to act synergistically and the occurrence of these alleles together increased the risk of developing SLE by at least 40-fold. It is possible that the *IL10G* susceptibility alleles identified in the study of SLE by Eskdale *et al.* (1997) may interact with other loci which influence aspects of the immune response related to the function of IL-10.

When the GEOP population was split according to smoking status, the trend towards a reduction in allele nine and an increase in allele 13 in the GEOP patients was found to be due to the smoking group (Figure 4.8). It has been demonstrated that smoking can have profound effects on the immune response in periodontitis (section 1.7.6). In addition, smoking may influence subgingival infection by specific periodontal pathogens (Zambon *et al.*, 1996a). A general consensus exists to the effect that some autoimmune diseases may be initiated by infection (Baines and Ebringer, 1992). It is possible therefore that smoking interacts with the *IL10* genotype to exacerbate the destruction seen in patients with GEOP. Alternatively, the increased IL-10 levels in the gingivae may normally be protective in nature (Mertz *et al.*, 1994). In this case smoking may interact in a deleterious manner with other aspects of the immune or inflammatory response and override the anti-inflammatory effect of Il-10. The *IL10* locus may be in linkage disequilibrium with another immune response locus situated nearby on chromosome one. Unfortunately no comparison between smoking status and distrubution of *IL10G* alleles could be made between EOP patients and controls, since no demographic data were available for the control population. However, the trend observed in the patient group is worthy of further investigation.

4.4.4 Concluding Remarks

As previously mentioned, EOP constitutes a group of diseases which may be genetically and environmentally heterogeneous. Although overriding evidence now exists for a genetic predisposition to EOP, unravelling the detail at the gene level is a complicated and time-consuming process. Within populations of patients subgroups very probably exist with different aetiologies of disease, increasing the difficulty of finding associations between genetic markers and GEOP. One of the major criticisms of studies of candidate genes in periodontitis is the lack of sample size (Kornman *et al.*, 1997; Galbraith *et al.*, 1998; Gore *et al.*, 1998). This makes interpretation of significant results unreliable. The data presented in this report represented a population of 75 GEOP patients, which is a substantial cohort compared with previous studies. In addition, a careful and conservative statistical analysis has been undertaken to ensure no bias or over-reporting of the significance of the findings. However, the

results of this study will require further investigation and verification before any conclusions are reached concerning the elucidation of the genetic basis of GEOP.

Chapter 5 Clinical and Genetic Analysis of a Large Family with Generalised Early Onset Periodontitis

5.1 Introduction

Since the 1920s researchers have recorded histories of families with more than one individual affected by periodontitis (Hassell and Harris, 1995). However, most of the evidence for a genetic predisposition to EOP comes from segregation analyses performed over the last 20 years. The majority of reports have not defined the criteria used for diagnosis of the affected and unaffected family members. Clearly, a better understanding of the pathological and genetic processes involved in EOP would occur if a biochemical or cellular marker that could be assayed without age restrictions could be identified. Until such a marker is found, it is necessary to use reported histories in a methodical manner.

In a recent study, autosomal dominant inheritance and race-specific heterogeneity in EOP was proposed (Marazita *et al.*, 1994). This was a large-scale segregation analysis using 100 nuclear families ascertained through 104 probands with EOP. The authors concluded that for each subset, the most likely mode of inheritance was autosomal dominant in both black and non-black families, with 73% penetrance in blacks and 70% in non-blacks. Because many of the probands' relatives were over 35 years of age when first examined, the study clinicians reviewed the radiographs and dental histories of all the individuals concerned. Any AP or edentulous subject with more than a 50% chance of having had EOP was re-categorised as affected. In this report, the criteria for reviewing an individual's radiographs and dental history were not documented. It is essential to adopt strict criteria in reviewing histories. This avoids major errors in the clinical and genetic analyses by over or under-estimating disease.

Both X-linked dominant and autosomal recessive inheritance of EOP have been proposed in earlier studies (Melnick, Shields and Bixler, 1976; Saxén, 1980b; Saxén and Nevanlinna, 1984; Page *et al.*, 1985; Spektor, Vandesteen and Page, 1985; Beaty *et al.*, 1987; Long *et al.*, 1987; Boughman *et al.*, 1988). The X-linked dominant theory is

based on a high female:male ratio among affected individuals, and lack of father-to-son transmission (Benjamin and Baer, 1967; Melnick, Shields and Bixler, 1976; Spektor, Vandesteen and Page, 1985). The preponderance of females in genetic studies of EOP is probably due to an ascertainment bias (Saxén, 1980b; Beaty *et al.*, 1987; Hart *et al.*, 1991). In addition, only a few fathers have participated in studies of EOP (Boughman *et al.*, 1988; Hart *et al.*, 1992). Earlier studies were based on an average of 25 pedigrees, which is a relatively small number. However, even in the recent large-scale study of 100 families there were almost half as many fathers compared with mothers who took part (Marazita *et al.*, 1994).

One group of investigators (Long *et al.*, 1987) concluded that an autosomal recessive model fitted significantly better than an X-linked dominant one, but they did not test an autosomal dominant model (Marazita *et al.*, 1994). Others (Beaty *et al.*, 1987; Boughman *et al.*, 1988) also found the best fitting model to be an autosomal recessive one but identified problems with the upper age-limit for diagnosing EOP. Marazita *et al.* (1994) analysed a large number of families compared with earlier studies of EOP and favoured an autosomal dominant mode of inheritance with reduced penetrance.

The study reported here presents a method for current and retrospective diagnosis of relatives of EOP probands when the ideal clinical data is not available. The index developed was then applied to the segregation analysis of a large pedigree with GEOP. In addition, the power of the large pedigree to detect linkage was investigated.

5.2 Materials and Methods

5.2.1 Ascertainment and Diagnosis of the Proband.

The proband was a patient who first presented at Glasgow Dental Hospital in September 1994. A full medical history was recorded to rule out any condition that might predispose to periodontal disease.

The clinical diagnosis of the proband used the classification for GEOP already described in section 2.3.1 (Hart *et al.*, 1991).

5.2.2 Diagnosis of Family Members - Assessment Procedures

Comprehensive medical and social histories were recorded for all relatives included in the analysis.

The full mouth periodontal charting is the most accurate method available of diagnosis of periodontitis. Measurements of attachment level and probing depth are recorded at six points round each tooth. Using this method, three categories were defined according to reliability of diagnosis of EOP (Table 5.1). The extent and severity of disease for age was also taken into account. Relatives fewer than 25 years of age with at least four teeth exhibiting $\geq$ 5 mm AL were classified as definitely affected. Individuals between 25 and 29 years old required at least six teeth to be placed in this category. Family members falling into the "probably affected" and "possibly affected" groups required the same number of teeth in each age group but showing AL of $\geq$ 4 mm and $\geq$ 3 mm, respectively.

Information from the clinical examination that stopped short of a full periodontal charting (Table 5.2) and from the reported case histories and anamnestic information (Table 5.3) was carefully analysed according to diagnostic value and attributed points. Age was also taken into consideration in the analysis. The total number of points scored by each individual was then compared with the values allocated, according to severity and extent of disease, from full mouth chartings (Table 5.1). This was in order to determine in which of the three categories an individual should be placed. A diagnostic flow diagram is illustrated in Figure 5.1.

A summary of the methods used for retrospective diagnosis of the proband's relatives is shown in Table 5.4. One family member, who was available for a full periodontal assessment but was 38 years old when he was examined, had interdental AL of between five and 14 millimetres around half of his remaining teeth.

Three models with different degrees of diagnostic reliability for GEOP were considered. A pedigree of the large family was constructed showing all three categories.

Table 5.1 **Diagnostic categories for genetic analysis of family members (up to 35 years of age).** Assessed using full mouth periodontal chartings – number of teeth required in each age group with a given level of AL.

AL (mm)	Age (years)			Points	Category
	< 25	25-29	30-35		
≥ 5 mm	4	6	8	5	definitely affected
≥ 4 mm	4	6	8	4	probably affected
≥ 3 mm	4	6	8	3	possibly affected

Table 5.2 **Any documented clinical evidence of periodontal disease (when aged < 45 years).**

Signs of disease		Points
Radiographic BL ≥ 1/3 root length on ≥ 2 teeth		1
One sextant with at least one tooth with a pocket of ≥ 5 mm measured apically from the ACJ		1
One sextant with at least one tooth with a pocket of ≥ 3.5 mm measured apically from the ACJ		½
Interproximal recession ≥ 3 mm on more than one tooth		1
Five missing teeth (excluding third molars)		1
Age of patient at examination -	20s	2
	30s	1
	40s	0

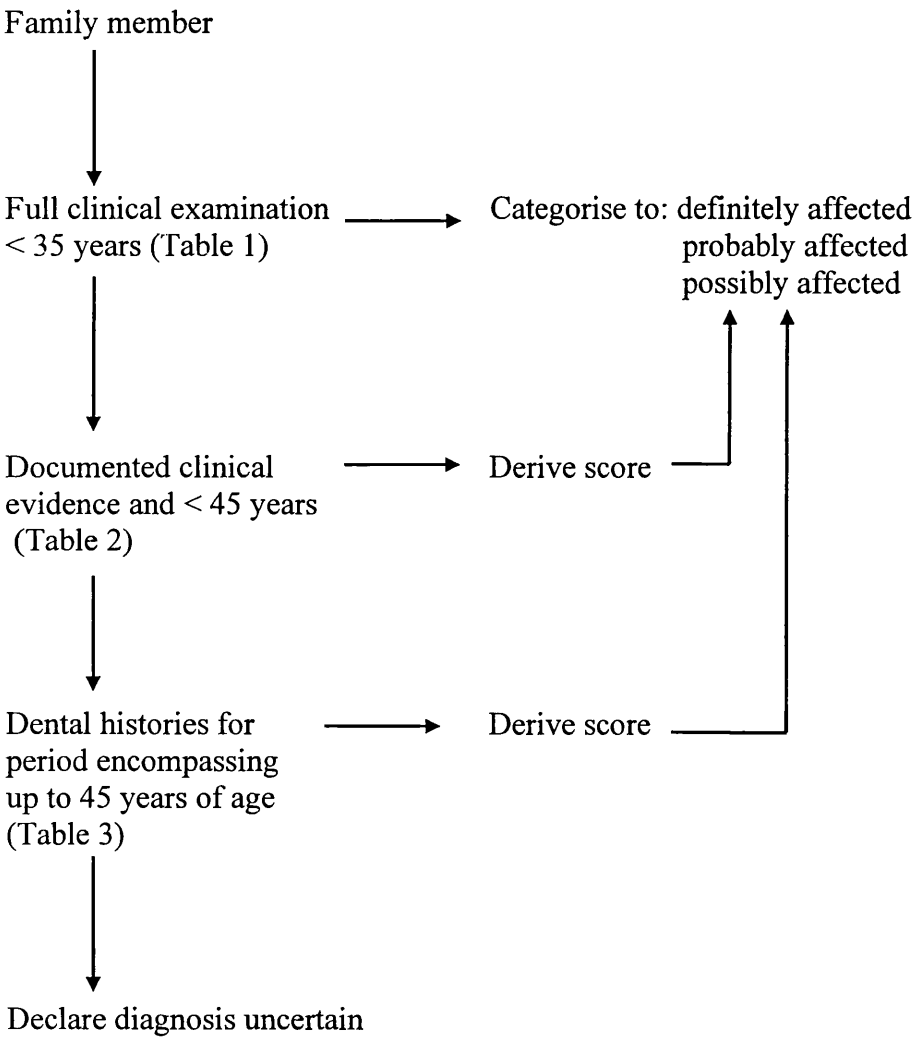
Table 5.3 **Evidence of periodontal disease - reported histories (for period encompassing up to 45 years of age).**

Symptoms		Points
Pyorrhoea (periodontal disease)/loose teeth		1
Bleeding on brushing		1
Multiple tooth loss without decay		1
Recurrent abscesses/bad taste/bad breath		1
Teeth appearing longer/spaced/exposed roots (sensitivity)		1
Age of reported tooth loss -	20s	3
	30s	2
	40s	1

Table 5.4 **Summary of methods used for current and retrospective diagnosis of relatives of large kindred - number of relatives in each group.**

Age	**Full mouth charting** (Table 5.1)	**Documented clinical evidence** (Table 5.2)	**Reported histories** (Table 5.3)
< 35	3	8	0
35-45	1	3	3
≥ 45	0	1	13

Figure 5.1 Diagnostic flow diagram.



Smoking status was measured in pack years. For affected individuals, the period of smoking was measured up until the age of diagnosis. In edentulous subjects, this was taken to be the age at which tooth loss occurred. In unaffected individuals, the number of pack years was calculated up to 35 years of age.

5.2.3 Validation of the Points System

The validity of the 'points system' was assessed. Diagnoses of individuals classified using full mouth periodontal chartings (Table 5.1) were compared with the results obtained using documented clinical evidence (Table 5.2) or reported histories (Table 5.3). The data for 12 relatives of EOP probands, including four relatives of the large kindred, was used for the comparison of Table 5.1 and Table 5.2. The documented clinical evidence was standard clinical information recorded during an initial visit to Glasgow Dental Hospital. Full periodontal chartings were then recorded at an interval of between two days and two months following the primary visit, apart from one individual who did not attend to have the charting recorded for 14 months.

Eight patients, who had lost multiple teeth and had removable dentures, were assessed using specific criteria from reported histories (Table 5.3). These diagnoses were then compared to the results obtained using previously recorded clinical data (Table 5.1) or, failing this, using documented clinical evidence (Table 5.2).

5.2.4 Statistical Methods

5.2.4.1 Validation of the points system

Kappa values were calculated using the SPSS statistical package.

5.2.4.2 Segregation analysis

Due to the high percentage of affected individuals in generations II and III, it was clear that the mode of inheritance of the disease trait in this family was not autosomal recessive. Autosomal dominant and X-linked dominant models were considered using

the chi-squared test. Individuals who had married into the family were not included in the segregation analysis.

5.2.4.3 Analysis of the power of the pedigree to detect linkage

Simulations were performed using the SIMLINK program (Boehnke, 1986; Ploughman and Boehnke, 1989). This program was used because the ultimate aim of this project is to carry out linkage analysis of the large kindred. The SIMLINK program is designed to estimate the power of a linkage study. It calculates the probability of detecting linkage for a given recombination fraction and indicates whether the pedigree provides sufficient information to demonstrate linkage. Recombination is the formation of new combinations of linked genes by crossing over between loci. The recombination fraction (θ) is the fraction of meiotic events that show a recombination between two loci. The program assumes Hardy-Weinberg equilibrium and no chromosomal interference. It employs the program MENDEL (Lange, Weeks and Boehnke, 1988) to calculate likelihoods. Replicates of each pedigree are simulated by SIMLINK for a given recombination fraction. MENDEL calculates a maximum lod score for each of the desired number of replicates (250 in the present case). The lod score is a base 10 logarithm of the odds favouring linkage. A lod score of +3 (1000:1 odds) is taken as proof of linkage; a score of -2 (100:1 odds against) indicates no linkage. SIMLINK uses the replicates to estimate the expected maximum lod score (and its standard error). It also derives the probability that a maximum lod score will exceed a particular value. Furthermore, maximum lod scores can be estimated for fixed marker distances when no linkage is assumed. This procedure permits the estimation of exclusion regions (over which the lod score is less than -2.0).

The SIMLINK program was applied to the pedigree with the three categories of GEOP considered separately and assuming different allele frequencies. For the purposes of the analysis the category with the least stringent diagnostic criteria (possibly affected) included the largest number of affected relatives. Therefore, the three groups consisted of:

1. all possibly affected cases (including probably and definitely affected individuals);

2. all probably affected cases (including definitely affected individuals);

3. all definitely affected cases.

The model assumed 75% penetrance up to 25 years of age and 97% thereafter. Penetrance is the observable expression of the mutant gene. The existence of two unrelated affected spouses is difficult to reconcile with the low frequency of the disease. In order to take account of the possible effect of environmental factors on the expression of GEOP in this pedigree, simulations were carried out with increasing phenocopy rates. A phenocopy is a phenotype due to environmental factors that mimics a genetically determined trait.

5.2.4.4 Analysis of smoking as a contributory factor

Analysis of variance was performed to estimate the relationship between smoking and GEOP. Adjustments were made in the analysis for the differences in smoking level between the six families of the siblings in generation II.

5.3 Results

5.3.1 Validation of the Points System

A comparison of the diagnoses of individuals from full mouth periodontal chartings (Table 5.1) with the results that would have been obtained if only documented clinical evidence (Table 5.2) had been available, achieved a kappa value of 0.8. This value indicates excellent agreement between these scoring methods (Fleiss, 1981). In all cases where reported histories (Table 5.3) were compared to the results obtained using previously recorded clinical data (Table 5.1) or, failing this, documented clinical evidence (Table 5.2), there was complete agreement between the scoring methods.

5.3.2 Segregation Analysis

The kindred consisted of 14 definitely affected, three probably affected, two possibly affected and 14 unaffected members (Figure 5.2). There were seven individuals for whom a diagnosis could not be made for one reason or another. Included in the analysis were two definitely affected spouses who had married into the family.

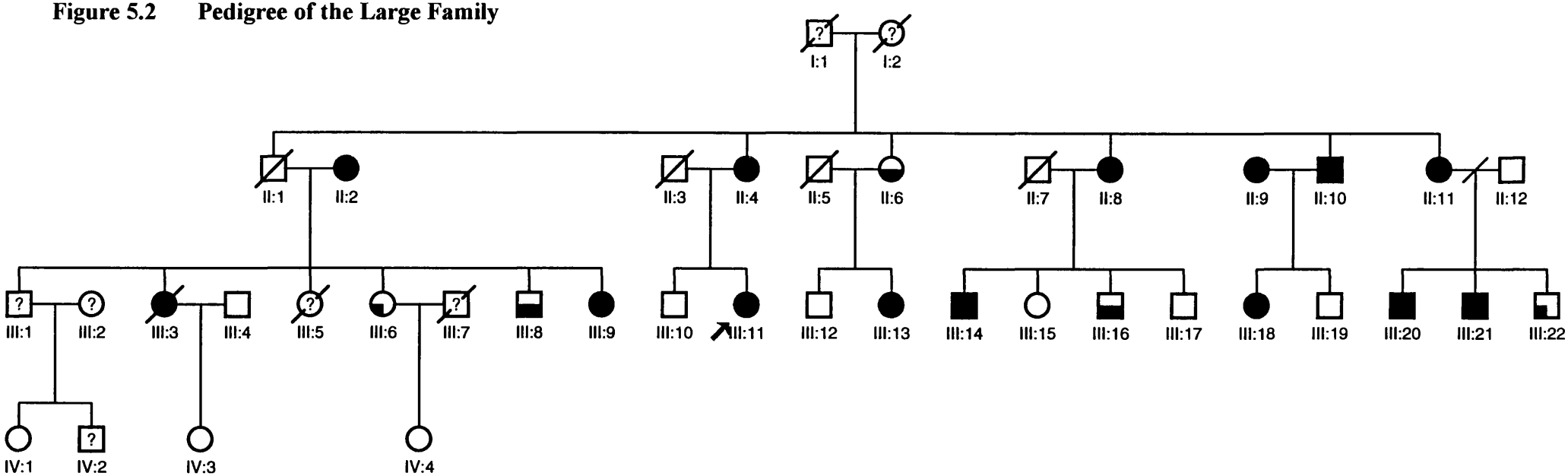
Assuming an autosomal dominant model (Table 5.5), of the 12 at risk male individuals, the expected number of affected subjects for the definitely affected category was 6.25, and unaffected subjects was 5.75. Among the 14 at risk females, 7.00 were expected to be affected and 7.00 unaffected. For the probably affected and possibly affected categories, the expected figures for males were 6.75 affected and 5.25 unaffected, and for females 7.50 affected and 6.50 unaffected. The small bias toward affected individuals in the expected category was due to the family where both parents were affected. There was no significant difference between the expected and observed values for the autosomal dominant model (Table 5.5).

Assuming an X-linked dominant model (Table 5.6) for the definitely affected category, out of the 12 males, the expected number of both affected and unaffected individuals was 6.0. For the 14 females, 7.0 were affected and 7.0 unaffected. For the probably and possibly affected categories, the expected figures for males were 6.5 affected and 5.5 unaffected and for females 7.5 affected and 6.5 unaffected. There was no significant difference between the expected and actual values for the X-linked dominant model (Table 5.6).

5.3.3 Simulation Results

The simulations were carried out assuming autosomal dominant inheritance since most studies of EOP have favoured autosomal inheritance. Table 5.7 shows the results of simulations in which the markers were placed at a fixed distance apart; the maximum multipoint lod scores are estimated for the trait at a number of positions between adjacent markers. In this analysis, a phenocopy rate of 1% was assumed. The estimates of the lod scores were above 3.0 for all positions of the trait gene and for all categories of disease. The probability of a maximum lod score of 3.0 (Table 5.8) at a

Figure 5.2 Pedigree of the Large Family



- unaffected male
- deceased unaffected female
- definitely affected female
- male of uncertain diagnosis
- probably affected female
- divorced unaffected male and female
- possibly affected male

Table 5.5 Autosomal dominant model.

Definitely affected

	Affected (expected)	**Unaffected (expected)**	**Total**
Male	4 (6.25)	8 (5.75)	12
Female	8 (7.00)	6 (7.00)	14
Total	12	14	26

$\chi^2 = 1.976$ with 2 degrees of freedom $p = 0.372$

Probably affected (including severely affected)

	Affected (expected)	**Unaffected (expected)**	**Total**
Male	6 (6.75)	6 (5.25)	12
Female	9 (7.50)	5 (6.50)	14
Total	15	11	26

$\chi^2 = 0.836$ with 2 degrees of freedom $p = 0.638$

Possibly affected (including severely and probably affected)

	Affected (expected)	**Unaffected (expected)**	**Total**
Male	7 (6.75)	5 (5.25)	12
Female	10 (7.50)	4 (6.50)	14
Total	17	9	26

$\chi^2 = 1.816$ with 2 degrees of freedom $p = 0.403$

Table 5.6 X-linked dominant model.

Definitely affected

	Affected (expected)	**Unaffected** (expected)	**Total**
Male	4 (6.0)	8 (6.0)	12
Female	8 (7.0)	6 (7.0)	14
Total	12	14	26

$\chi^2 = 1.619$ with 2 degrees of freedom $p = 0.445$

Probably affected (including severely affected)

	Affected (expected)	**Unaffected** (expected)	**Total**
Male	6 (6.5)	6 (5.5)	12
Female	9 (7.5)	5 (6.5)	14
Total	15	11	26

$\chi^2 = 0.730$ with 2 degrees of freedom $p = 0.694$

Possibly affected (including severely and probably affected)

	Affected (expected)	**Unaffected** (expected)	**Total**
Male	7 (6.5)	5 (5.5)	12
Female	10 (7.5)	4 (6.5)	14
Total	17	9	26

$\chi^2 = 1.878$ with 2 degrees of freedom $p = 0.391$

Table 5.7 Expected maximum multipoint lod score at true map distances (cM) - based on 250 simulations.

Map distance from one marker to mutant gene[a]	Multipoint lod score[b,c]		
	Possibly Affected + Probably Affected + Definitely Affected	Probably Affected + Definitely Affected	DefinitelyAffected
2	3.80 (±0.09)[d]	3.69 (±0.08)	3.66 (±0.08)
6	3.29 (±0.08)	3.35 (±0.09)	3.42 (±0.09)
10	3.21 (±0.09)	3.31 (±0.08)	3.29 (±0.08)
14	3.27 (±0.08)	3.22 (±0.09)	3.20 (±0.09)
18	3.83 (±0.08)	3.65 (±0.08)	3.69 (±0.08)
INFINITE	0.03 (±0.01)	0.01 (±0.01)	0.04 (±0.01)

[a]Two flanking markers at fixed 20cM map distance.

[b]Penetrance increasing from 0.75 to 0.97 at 25 years old.

[c]Phenocopy rate 0.01.

[d]Figures in parentheses are standard error of the mean.

Table 5.8 Estimated probability of a maximum multipoint lod score ≥ 3 for two markers at a fixed map distance (cM) – based on 250 simulations.

Map Distance from one Marker to Mutant Gene[a]	Probability[b,c]		
	Possibly Affected + Probably Affected + Definitely Affected	Probably Affected + Definitely Affected	DefinitelyAffected
2	0.70 (±0.03)[d]	0.73 (±0.03)	0.72 (±0.03)
6	0.60 (±0.03)	0.60 (±0.03)	0.60 (±0.03)
10	0.59 (±0.03)	0.60 (±0.03)	0.60 (±0.03)
14	0.62 (±0.03)	0.58 (±0.03)	0.58 (±0.03)
18	0.74 (±0.03)	0.68 (±0.03)	0.68 (±0.03)
INFINITE	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)

[a]Two flanking markers at fixed 20cM map distance.

[b]Penetrance increasing from 0.75 to 0.97 at 25 years old.

[c]Phenocopy rate 0.01.

[d]Figures in parentheses are standard error of the mean.

true marker recombination rate (Table 5.9) was at least 0.68, close to a flanking marker, and at least 0.59, at the midpoint between them. The probability of a type I error, i.e. obtaining a maximum lod score of 3.0 when there is no linkage was zero. The lod score was always below -3.78 when both flanking markers were considered to be unlinked (Table 5.10). A lod score of less than -2.0 allows exclusion of linkage for the whole interval between the markers (Table 5.11).

There was very little variation between lod scores for the three separate groups (Table 5.7). Therefore only the definitely affected group was re-examined using increasing phenocopy rates (Table 5.12). A phenocopy rate of 15% is consistent with two out of 14 affected individuals being phenocopies. The results at this rate still provide evidence of linkage (expected maximum lod score = 3.0). The probability of finding a lod score of more than or equal to 3.0 is greater than 0.5. In addition, a satisfactory negative lod score for two unlinked markers is also evident.

5.3.4 Results of Smoking Analysis

Analysis of variance of the effect of smoking on the occurrence of GEOP showed no evidence of an increasing trend in pack years with severity of disease ($p = 0.476$) (Table 5.13). Although all four non-smokers in generations III and IV were unaffected (Figure 2, III:15, IV:1,3,4), there were five severely affected individuals who had smoked less than 5 pack years (II:4, II:8, III:13, III:14, III:21) and two severely affected (III:3, III:9) against three unaffected subjects (II:1, II:11, III:4) who had smoked more than 15 pack years.

5.4 Discussion

There are no family studies of EOP published in the dental literature which precisely define the criteria used for the diagnosis of relatives, other than the proband. The study presented here has attempted to address this problem. It was essential that as many of the relevant family members as possible were included in the segregation analysis. Excluding individuals over the age of 35 years could result in a dominant form of the disease appearing to be recessive due to lack of parent-offspring

Table 5.9 **True map distances and recombination fractions between each marker and the mutant gene.**

Marker 1		Marker 2	
Distance (cM)	**Recombination fraction**	**Distance** (cM)	**Recombination fraction**
2	0.02	18	0.15
6	0.06	14	0.12
10	0.09	10	0.09
14	0.12	6	0.06
18	0.15	2	0.02
INF	0.50	INF	0.50

Table 5.10 Expected lod score for a pair of unlinked markers – based on 250 simulations.

Test map distance from marker to mutant gene[a]	Multipoint lod score[b,c]		
	Possibly Affected + Probably Affected + Definitely Affected	Probably Affected + Definitely Affected	DefinitelyAffected
2	-5.61 (±0.17)[d]	-5.76 (±016)	-5.58 (±0.17)
6	-4.12 (±0.13)	-4.22 (±0.13)	-4.08 (±0.14)
10	-3.83 (±0.12)	-3.90 (±0.13)	-3.78 (±0.13)
14	-4.17 (±0.13)	-4.23 (±0.13)	-4.13 (±0.14)
18	-5.75 (±0.17)	-5.80 (±0.17)	-5.72 (±0.18)

[a]Two markers at fixed 20cM map distance.

[b]Penetrance increasing from 0.75 to 0.97 at 25 years old.

[c]Phenocopy rate 0.01.

[d]Figures in parentheses are standard error of the mean.

Table 5.11 Approximate map distances (cM) at which the location score equals -2.0 (interpolated):

Marker 1: 20

Marker 2: 20

Proportion of interval excluded (%): 100

Table 5.12 **Expected maximum lod score, estimated probability, and expected lod score for unlinked markers, with increasing phenocopy rate – based on 250 simulations.**

Phenocopy rate (%)	Max lod score for 2 markers equidistant at 10cM	Probability of a lod score of ≥ 3.0 with 2 markers equidistant at 10cM	Lod score for a pair of un-linked markers
1	3.29 (±0.08)[a]	0.60 (±0.03)	-3.78 (±0.13)
10	2.98 (±0.09)	0.52 (±0.03)	-4.08 (±0.12)
15	3.00 (±0.09)	0.53 (±0.03)	-4.13 (±0.14)
20	2.89 (±0.09)	0.51 (±0.03)	-3.82 (±0.12)

[a]Figures in parentheses are standard error of the mean.

Table 5.13 **Number of relatives who smoked increasing levels of pack years and the mean pack years smoked for each diagnostic category.**

Pack years	Unaffected	Possibly Affected	Probably Affected	Definitely Affected
	(n = 14)	(n = 2)	(n = 3)	(n = 14)
0-5	8	0	3	5
5-10	3	2	0	3
10-15	0	0	0	4
≥15	3	0	0	2
Mean Pack Years	6.09	8.75	1.99	9.35

transmission. However, individuals under the age of 20 were not included; it was considered that the disease phenotype had not developed sufficiently at this age to reliably differentiate healthy from diseased subjects (Albandar *et al.*, 1997).

5.4.1 Clinical Analysis

An index was required for the current and retrospective diagnosis of relatives of EOP probands. The first stage in the development of such an index was to decide on the diagnostic criteria for the affected relatives under the age of 35, who were willing to allow a full periodontal assessment to be carried out (Table 5.1). Some investigators have used the same diagnostic criteria as for the proband (Hart *et al.*, 1991; Marazita *et al.*, 1994). However, in these studies a reduced threshold of clinical AL for the affected family members was applied. This was set at a minimum of 2 mm at affected molars and incisors in LEOP and a minimum of 3 mm in GEOP. It is suggested that these criteria are not stringent enough to distinguish between EOP affected individuals and AP subjects. AP also appears to be initiated during the teenage years (Clerehugh, Lennon and Worthington, 1990). Therefore, it was decided that AL of $\geq$ 5 mm, which is evidence of established disease, must be present in order for an individual to be classified as definitely affected by EOP. However, the age of the patient at examination i.e. between 20 and 35 years should be factored into the assessment. Fewer teeth with AL of $\geq$ 5 mm were required in the 25- to 29-year and the under 25-year age groups for the family member to be classified as definitely affected. The criteria used for any documented or clinical evidence of periodontal disease (Table 5.2) were carefully weighted. This was done as a conservative measure of the diagnostic value of each criterion, in reflecting the severity and extent of disease from full mouth periodontal chartings (Table 5.1). The age of the patient at examination up to 45 years was also taken into consideration.

The symptoms of the disease prior to, or the reasons for, tooth loss as reported in the histories (Table 5.3) are well established in the literature (Benjamin and Baer, 1967; Fourel, 1972; Jorgenson *et al.*, 1975; Melnick, Shields and Bixler, 1976; Saxén, 1980b). The age of reported tooth loss up to a limit of 45 years was also included and weighted in the analysis. Any individual who had suffered multiple tooth loss without

decay, between the ages of 35 and 45, and who reported other symptoms of the disease, almost certainly had periodontal disease (Allen, 1944). In addition, a family history of the disease made it more likely that an individual between 35 and 45 years who had documented clinical evidence of disease (Table 5.2) or a reported history of periodontal disease (Table 5.3) was affected by EOP.

5.4.2 Segregation Analysis

The results of the segregation analysis are consistent with a gene of major effect for GEOP inherited in either an autosomal dominant or an X-linked dominant manner. Genetic disorders having an X-linked dominant mode of inheritance are very rare. The majority of these traits are lethal in hemizygous males (McKusick, 1994; Connor and Ferguson-Smith, 1997). Furthermore, many previous family studies of EOP have indicated an autosomal pattern of inheritance (Saxén, 1980b; Saxén and Nevanlinna, 1984; Page *et al.*, 1985; Spektor, Vandesteen and Page, 1985; Boughman *et al.*, 1986; 1988; Beaty *et al.*, 1987; Long *et al.*, 1987; Hart *et al.*, 1992; Marazita *et al.*, 1994).

5.4.3 Analysis of the Power of the Pedigree to Detect Linkage

There was no systematic difference between the lod scores for the three categories of disease (definitely, probably and possibly affected) in the three-point simulations, using two flanking markers (Table 5.7). The SIMLINK analysis indicated that the pedigree provides sufficient information for a linkage study. Multipoint linkage would be able to confirm or reject linkage of a trait gene in the interval between markers, placed at an average separation of 20 cM. In addition there was no systematic difference between the lod scores for the three groups in two-point simulations, using a single marker (data not shown). It should be borne in mind that the penetrance levels used for this analysis were conservative.

The phenocopy rate was increased in order to take account of possible environmental aetiological factors. It was found that even with a phenocopy rate of 15% the pedigree would still be informative for linkage analysis.

5.4.4 Specific Environmental Risk Factors

It has been proposed that the two most important environmental risk factors for periodontitis are smoking and the presence of specific periodontal pathogens (Grossi *et al.*, 1994; 1995; Genco, 1996; Barbour *et al.*, 1997). It was not possible to carry out microbiological analysis because several of the affected relatives were edentulous. However, despite evidence for transmission of periodontal pathogens within families (Petit *et al.*, 1993a; Asikainen, Chen and Slots, 1996), this observation on its own is insufficient to account for the familial clustering seen in EOP (Boughman, Astemborski and Suzuki, 1992; van der Velden *et al.*, 1993; Tinoco, Sivakumar and Preus, 1998).

When smoking was considered as an independent variable, it was not found to be associated with GEOP in this family (Table 5.9). Of interest were the five relatives who were definitely affected by GEOP but were very light smokers (< 5 pack years). It has been shown that very light smokers (< 5 pack years) have no increased risk for periodontitis above non-smokers (Bergström, Eliasson and Preber, 1991; Grossi *et al.*, 1994; 1995; Martinez-Canut, Lorca and Magán, 1995). In addition there were three unaffected family members who were moderate to heavy smokers ($\geq$ 15 < 20 pack years). Therefore, it appears that genotype overrides any effect due to smoking in the manifestation of the disease in this family.

5.4.5 Concluding Remarks

An autosomal dominant hypothesis is proposed against a low background prevalence of the disease in the general population. Unfortunately no epidemiological studies exist for EOP in a Scottish population. However, a recent report of periodontal status in the USA between 1988 and 1991 in 7447 dentate individuals aged 13 years and older showed low prevalence of established disease in the younger age groups (Brown, Brunelle and Kingman, 1996). This study reported a prevalence of AL $\geq$ 5 mm of 03% in the 13-17 and 18-24 age groups and 6.4% in the 25-34 age group. Interpretation of these results must take into account the inclusion of LEOP, GEOP and IEOP in the data-set. Another recent survey of 5,849 individuals aged 18-34 years

found a prevalence of only 0.3% for GEOP where AL of ≥ 5 mm was present on eight teeth (Oliver, Brown and Löe, 1998). In neither of these studies was race considered separately for these age groups. Caucasians have a much lower reported prevalence of EOP than blacks and Hispanics (Saxby, 1984b; 1987; Ismail *et al.*, 1987; Löe and Brown, 1991). Therefore, these prevalence statistics may overestimate the levels of disease in Caucasians.

The large kindred studied here is unusual in present times because most of the family were born and remained within two neighbouring villages in South-West Scotland. The two affected spouses in generation II who married into the family also came from the same community. No evidence could be found of kinship between the spouses and the large family when their lineage was traced back through two generations. However, it is possible that the spouses were in fact distant relatives of the proband.

In conclusion:

1. A system has been developed which can be used for the diagnosis of relatives of EOP probands when ideal clinical data is not available.

2. The index has been carefully validated.

3. The work presented here supports the autosomal dominant theory of genetic transmission in GEOP.

4. This large kindred is suitable for a genetic linkage study with the aim of identifying the location of a susceptibility gene(s).

Chapter 6 General Conclusions

6.1 Summary and Conclusions

For many years heredity has been suspected to play a part in the aetiology of periodontal disease. The findings presented in this thesis contribute to the accumulating evidence for a genetic predisposition to EOP.

Major advances in the understanding of the pathogenesis of the periodontal diseases have come to light during the last decade. It is now possible to identify potential defects in aspects of the immune and inflammatory response, which may increase susceptibility to the pathogens in the subgingival microbiota. Using recently developed molecular genetic techniques these defects can be investigated at the gene level.

Analysis of genetic polymorphisms in the HLA complex and the IL.10 promoter region were undertaken. No significant associations were found between any of the candidate genes and EOP. However, trends were noted in the occurrence of alleles at the IL.10.G and TNFa microsatellites. When smoking was included as a covariate for the patient group, the trend seen at the IL.10.G locus was found to be due to the smokers. In addition, a significant association ($p = 0.047$) was found between the TNFa2TNFd4 allelic combination and GEOP, which had previously been identified as a disease genotype in Crohn's disease. These observations warrant further investigation. However, they also highlight the problems of examining candidate genes in EOP. Since EOP is a genetically and environmentally heterogeneous group of diseases, finding convincing associations with specific polymorphisms in populations of unrelated individuals is extremely difficult.

One of the problems of genetic model testing in families with an increased frequency of EOP is the confusion that exists between different research groups with regard to diagnosis of relatives of EOP probands. An index has been defined and validated which is suitable for the current and retrospective diagnosis of family members. The

application of this index to a large family with GEOP supports the autosomal dominant theory of inheritance of this disease.

6.2 Suggestions for Future Research

6.2.1 Candidate Genes

The transmission disequilibrium test and the affected sib-pair design are other ways of studying candidate genes in large populations. These methods ensure that the disease population and the controls are genetically homogeneous and are useful for investigating diseases such as periodontitis, which have a multifactorial aetiology.

Another possible method of unravelling the role of candidate genes in periodontitis is to study the frequency of specific polymorphisms in a control group consisting of individuals who are resistant to disease.

6.2.1.1 Proinflammatory cytokines

It would be interesting to analyse a control group for the IL.10.G locus including smoking as a covariate. In so doing any possible association between GEOP smokers and the combined IL.10.G9 and G13 genotype would be revealed.

Because of the problems of heterogeneity of EOP described above, it would be worthwhile investigating IL-10 levels in the gingival tissues of patients and controls in combination with the IL.10 microsatellites. Eskdale *et al.* (1998a) recently demonstrated that IL-10 secretion can vary according to different haplotypes within the IL.10 promoter region. This approach would be a useful way of defining groups of individuals who are more homogeneous. It could also be applied to the analysis of TNF and other cytokine polymorphisms in EOP.

6.2.1.2 Anabolic cytokines

Research into the genetic basis of periodontal disease could be usefully directed towards investigating the anabolic cytokines. These include PDGF, fibroblast growth

factor, insulin-like growth factor, transforming growth factors and the bone morphogenic proteins (Offenbacher, 1996). Many cell types including monocytes, platelets and fibroblasts secrete these molecules during tissue repair. Fibroblasts, periodontal ligament cells and bone precursor cells are attracted to areas of growth factor secretion and stimulated to undergo mitosis and proliferate. Growth factors may also promote the secretion of collagen and glycosaminoglycans by their action on maturing connective tissue mesenchymal cells. Variation in growth factor expression exists between different individuals. For example diabetics and smokers have a reduced capacity for monocytic growth factor secretion. This finding may be important since both smokers and diabetics demonstrate impaired wound healing.

6.2.1.3 Regulation of proinflammatory cytokines

Without doubt the proinflammatory cytokines IL-1 and TNF play a key role in precipitating the destructive processes seen in periodontal disease. However, important regulatory molecules such as soluble receptors and IL-1ra have also been identified. Howells (1995) proposed that periodontitis may be due to decreased levels of these cytokine regulators rather than overproduction of IL-1 or TNF. An increased frequency of allele 2 in the IL-1ra gene (IL.1RN) has been found in individuals with increased production of IL-1ra protein and decreased production of IL-1α by monocytes (Danis *et al.*, 1995). However, the same allelic polymorphism has also been associated with a number of chronic inflammatory diseases including ulcerative collitis and SLE, when the reverse might be expected (Mansfield *et al.*, 1994; Blakemore *et al.*, 1994). A subsequent study in patients with inflammatory bowel disease found a significant association with a combined genotype (an increased frequency of IL.1RN2 and a reduced frequency of allele 2 of IL.1B +3953) and both Crohn's disease and ulcerative collitis (Bioque *et al.*, 1995). Since allele 2 of IL.1B +3953 represents a high secretor phenotype the authors suggested that the combined genotype might interact to influence the biological outcome of chronic inflammatory bowel disease. Alternatively, this haplotype could be a genetic marker in linkage disequilibrium with other alleles nearby. A study of AP patients (mild, moderate and severe) found no association between IL.1RN and severity of disease (Kornman *et al.*,

1997). An investigation of phenotypic and genetic associations between cytokines, cytokine regulators and EOP might yield interesting results.

6.2.2 Family Studies

The ultimate aim of the clinical and genetic analysis of the large kindred presented here is to carry out a family linkage study. During the process of identifying the large family a number of nuclear families with a history of EOP were discovered (Appendix Three). It would be worthwhile to continue collecting data from smaller pedigrees since they could be useful for future linkage analysis and investigation of genetic polymorphisms as already mentioned above.

6.3 Concluding Remarks

The future identification of genetic markers of susceptibility to periodontal disease could have wide-ranging health and economic implications. Targeting preventive measures at individuals most likely to benefit from them, instead of employing a broad-based approach to the treatment of periodontitis would preserve valuable financial and healthcare resources. During the last five years a number of researchers have suggested that periodontal disease may predispose to atherosclerosis and coronary heart disease (Beck *et al.*, 1996; Seymour and Steele, 1998). If these initial findings are corroborated, the identification of risk markers for periodontal disease will become increasingly important in the prevention and treatment of cardiovascular disorders.

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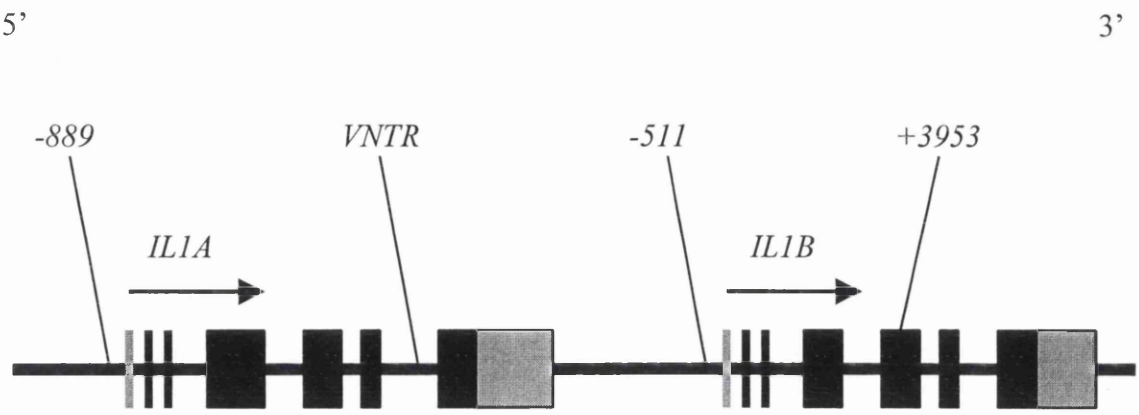
Appendix 1 DNA Analysis of *IL1* Polymorphisms in Generalised Early Onset Periodontitis

A1.1 Introduction

Cytokines are regulatory proteins of low molecular weight, which are active in very small amounts (femtomolar to picomolar concentrations). They transmit information between cells and form a complex network of interactions. Most cytokines are pleotropic. They may act synergistically or may inhibit the action of other cytokines. They are produced by activated cells and mainly exert their effects locally by binding to high affinity receptors on the surface of a variety of cell types. Stable interindividual differences in cytokine secretion patterns have been reported (Mølvig *et al.*, 1988). It has also been demonstrated that allelic variation at cytokine loci, and in genes regulating their expression, may affect cytokine responses between individuals (Pociot *et al.*, 1991; 1993; Messer *et al.*, 1991; Derkx *et al.*, 1995; Bailly *et al.*, 1996; Eskdale *et al.*, 1998; Hurme and Santtila, 1998; Santtila *et al.*, 1998). In addition, associations between polymorphisms of candidate cytokine genes and certain diseases have been documented (Pociot *et al.*, 1991; 1993; McGuire *et al.*, 1994; Wilson *et al.*, 1994; Eskdale *et al.*, 1997).

The proinflammatory cytokine, IL-1, plays a central role in the pathogenesis of many autoimmune and infectious diseases including periodontitis (Roberts *et al.*, 1997). The genes encoding IL-1α and IL-1β lie on the long arm of chromosome two, approximately 70 kilobases apart (Figure A1.1). They are also closely linked with the gene for their endogenous inhibitor IL-1 receptor antagonist (*IL1RN*). Variations in *in vitro* production of IL-1 between individuals suggests a hereditary interindividual influence (Mølvig *et al.*, 1988; Endres *et al.*, 1989). It has been suggested that polymorphisms influencing transcription of IL-1α and IL-1β may give rise to differential production of these cytokines. Bi-allelic polymorphisms within the promotor regions of the *IL1* genes (*IL1A –889*, *IL1B –511*) have been identified (di Giovine *et al.*, 1992; McDowell *et al.*, 1995). In addition, variable number of tandem

Figure A1.1 Diagrammatic representation of the human *IL1A* and *IL1B* genes



The position of the following biallelic polymorphisms are illustrated: *IL1A –889* (*Nco*I) (Tarlow *et al.*, 1993); *IL1B –511* (*Ava*I) (di Giovine *et al.*, 1992); *Il1B +3953* (*Taq*I) (Pociot *et al.*, 1992). In addition a polymorphism due to a variable number of the 46 bp tandem repeat (*VNTR*) within intron six of *IL1A* is also illustrated (Bailly *et al.*, 1993)

Adapted from Nicklin *et al.* (1994), Furutani *et al.* (1986) and Bensi *et al.* (1987).

repeats (*VNTR*) (section 5.4.1) in the sixth intron of the *IL1A* gene and the second intron of the *IL1RN* gene and a further bi-allelic polymorphism in the fifth exon of the *IL1B* gene (*IL1B +3953*) have been described (Pociot *et al.*, 1992; Tarlow *et al.*, 1993; Bailly *et al.*, 1993). Allele two of *IL1B +3953* has been associated with significantly higher secretion of IL-1β in individuals homozygous for this allele (Pociot *et al.*, 1992). Associations have also been found between specific alleles of these polymorphisms and a number of chronic inflammatory diseases (Pociot *et al.*, 1992; Mansfield *et al.*, 1994; Blakemore *et al.*, 1994; McDowell *et al.*, 1995). In one study of inflammatory bowel disease, a significantly higher frequency of allele two of the *VNTR* in the second intron of *IL1RN* together with allele one of *IL1B +3953* was found in patients with both ulcerative colitis and Crohn's disease (Bioque *et al.*, 1995). More recently, it has been suggested that these polymorphisms may be markers of disease severity rather than susceptibility (Nemetz *et al.*, 1999; Jouvenne *et al.*, 1999; Schrijver *et al.*, 1999).

A recent paper has indicated an association between a composite genotype of allele 2 of the *IL1B +3953* gene and allele 2 of the *IL1A –889* gene and the severe form of AP (Kornman *et al.*, 1997). The patients in this study were Caucasian and of north European origin. The association was only found in non-smokers. The aim of the present study was to investigate the *IL1A –889* (McDowell *et al.*, 1995) and *IL1B +3953* (Pociot *et al.*, 1992) polymorphisms in Caucasian EOP patients.

A1.2 Materials and Methods

A1.2.1 Patient Selection

Fifty-six patients were recruited to this study based on the criteria described previously (section 2.3.1). There were seven patients recruited to the study who were over the age of 35 years when they presented in the periodontal department of Glasgow Dental Hospital and School. The inclusion criteria were as described in section 3.2.1.

A1.2.2 Control Group

The control group included 56 volunteers from the staff of Glasgow Dental Hospital with no evidence of GEOP. The same selection criteria were applied as described in section 3.2.2. Demographic data for the patient and control groups are presented in Table A1.1.

A1.2.3 DNA Purification

DNA separation was performed as described in section 3.2.3. DNA integrity was checked and DNA quantitated using agarose gel electrophoresis as previously described (sections 2.3.5 and 3.2.3).

A1.2.4 Screening of Subjects for the *IL1A* and *IL1B* Polymorphisms

A1.2.4.1 PCR

For the PCR reactions the following synthetic specific oligonucleotide primers were used (Kornman *et al.*, 1997):

IL1A –899 primer 1: 5'-AAG.CTT.GTT.CTA.CCA.CCT.GAA.CTA.GGC-3'
primer 2: 5'-TTA.CAT.ATG.AGC.CTT.CCA.TG-3'; both at a concentration of 0.6μm.

IL1B +3953 primer 1: 5'-CTC.AGG.TGT.CCT.CGA.AGA.AAT.CAA.A-3'
primer 2: 5'-GCT.TTT.TTG.CTG.TGA.GTC.CCG-3'; both at a concentration of 0.2μm.

A master mix containing all the reagents minus the sample DNA was set up on ice. The upper master mix contained the primers. The total volume of each reaction was 100μl. The contents of the lower master mixes are shown in Table A1.2. One microlitre of sample DNA was added to the upper layer of each reaction. The details of "hot start" PCR have been described previously (section 3.2.4.1). The thermocycling conditions were as follows:

IL1A: An initial denaturation step at 94°C for five minutes was followed by 45 cycles of denaturation at 94°C for one minute, annealing of primers at 50°C for one minute

Table A1.1 Demographic data for analysis of *IL1* polymorphisms

	Total[a]	Male[a]	Female[a]	Smokers[a]	Non-smokers[a]	Mean age[b]
Patients	56 (100)	23 (41)	33 (59)	27 (48)	29 (52)	31.36 (16-40)
Controls	56 (100)	23 (41)	33 (59)	16 (29)	40 (71)	34.39 (21-61)

[a] Numbers of individuals in each group; percentages in parentheses.

[b] Mean age and range in parentheses, in years.

Table A1.2 Lower master mixes

1. Lower master mix *IL1A -889*

Reaction Component	Concentration in final volume	Vol per 100 µl (per reaction)
Mg Free Buffer (10x)	1 x	10.0 µl
50mM $MgCl_2$	1mM	2.0 µl
Dynazyme/Taq polymerase	2 units	0.4 µl
dNTPs (20mM)	0.2mM	1.0 µl
MBG H_2O	—	36.6 µl

2. Lower master mix *IL1B +3953*

Reaction Component	Concentration in final volume	Vol per 100 µl (per reaction)
PCR Buffer (10x)	1 x	10.0 µl
50mM $MgCl_2$	1mM	2.0 µl
Dynazyme/Taq polymerase	2 units	0.4 µl
dNTPs (20mM)	0.2mM	1.0 µl
MBG H_2O	—	36.6 µl

and primer extension at 72°C for one minute. A final extension step at 72°C for 10 minutes completed the cycling.

IL1B: Two cycles of denaturation at 95°C for two minutes, annealing of primers at 67.5°C for one minute and extension at 74°C for one minute was followed by 35 cycles at 95°C for one minute, 67.5°C for one minute and 74°C for one minute. Finally three cycles of 95°C for one minute, 67.5°C for one minute and 74°C for five minutes completed the reaction.

The outcome of each PCR was confirmed by electrophoresis on a 2% agarose gel as described in section 3.2.4.1.

A1.2.4.2 Purification of PCR products

The DNA was precipitated as described previously in section 3.2.4.2.

A1.2.4.3 Restriction endonuclease digestion of PCR products

IL1A: Amplified DNA was digested with *Nco*I in a volume of 20 µl. Six microlitres of sample DNA were added to the digestion mixture which comprised 2 µl of recommended 10x restriction enzyme buffer (supplied by the enzyme manufacturer), 0.4 µl *Nco*I (10 units/µl), 0.4 µl 2mM spermidine and MBG water to 20 µl. Digestion reactions were thoroughly vortex-mixed and incubated at 37°C for a minimum of three hours.

IL1B: Amplified DNA was digested with *Taq*I in a volume of 20 µl. Six microlitres of sample DNA were added to the digestion mixture which comprised 2 µl of recommended 10x restriction enzyme buffer (supplied by the enzyme manufacturer), 1.0 µl *Taq*I (10 units/µl), 0.4 µl 2mM spermidine and MBG water to 20 µl. Digestion reactions were thoroughly vortex-mixed and incubated at 65°C for a minimum of three hours.

The digested *IL1A* and *IL1B* products were subjected to electrophoresis on 4.0 % agarose gels as described previously (section 2.3.5).

A1.2.4.4 Contamination controls

The same protocol was applied to the PCR and post-PCR procedures as described in section 3.2.4.4.

A1.2.5 Statistical Analysis

Statistical analysis was performed using the CLUMP programme devised by Sham and Curtis (1995) (section 4.2.6). This programme was used so that the three genotype frequencies (1/1 homozygotes, heterozygotes and 2/2 homozygotes) as well as individual allele frequencies could be analysed at the same time.

Table A1.3 Stock solutions and buffers

The stock solutions and buffers were as previously described in Chapters Two and Three with the addition of:

Mg Free Buffer

10mM Tris HCl

50mM KCl

0.1% Triton®X-100

50mM $MgCl_2$

Diluted to desired concentration in reaction mixture (1.0mM).

A1.3 Results

A1.3.1 Purified Genomic DNA

DNA integrity was tested as previously described (section 3.3.1, Figure 3.2).

A1.3.2 PCR Products

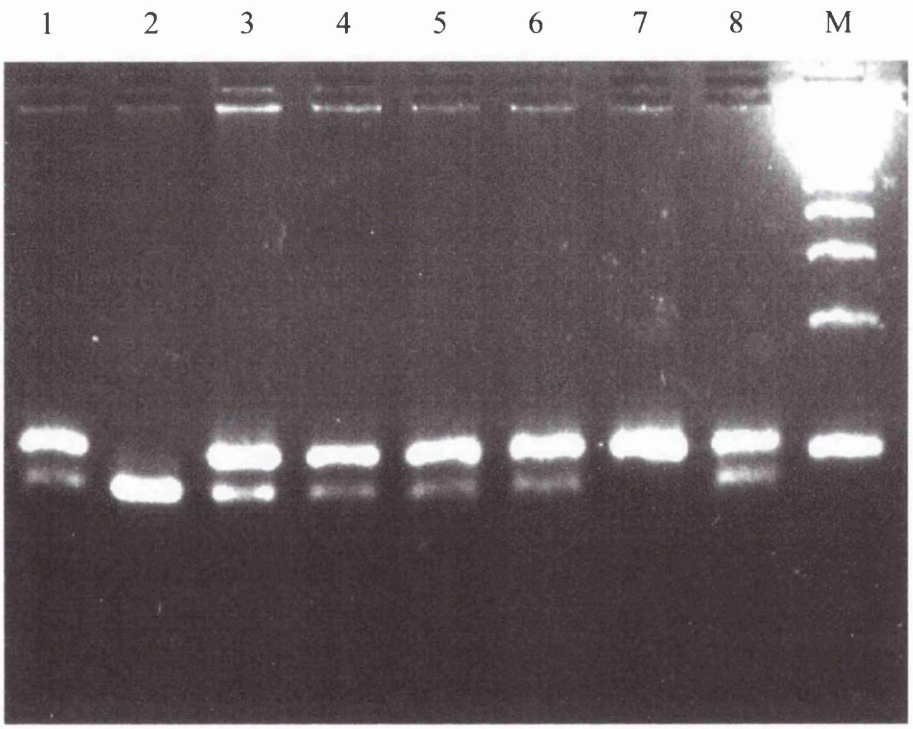
To confirm amplification of the 99bp (*IL1A –889*) and 194bp (*IL1B +3953*) products, samples were run on 2.0 % agarose gels. A 100-bp marker was used to verify the size of the products.

A1.3.3 Digestion of PCR Products

In order to investigate the frequency of alleles of the *IL1A –889* and *IL1B +3953* polymorphisms, in patients and controls, digested PCR products were run on 4.0 % agarose gels. Figures A1.2 and A1.3 show photographs of typical gels. Fragments of 99 bp (allele 2) and approximately 83 bp (allele 1) are seen for patients and controls heterozygous for *IL1A –889*. For the *IL1B +3953* polymorphism fragments of 182 bp (allele 2) and 97 and 85 bp (allele 1) are visible for heterozygotes. In this study, the expected 16 bp (*IL1A –889*) and 12 bp (*IL1B +3953*) fragments described by Kornman *et al.* (1997) were not seen, probably because of the small size of the fragments.

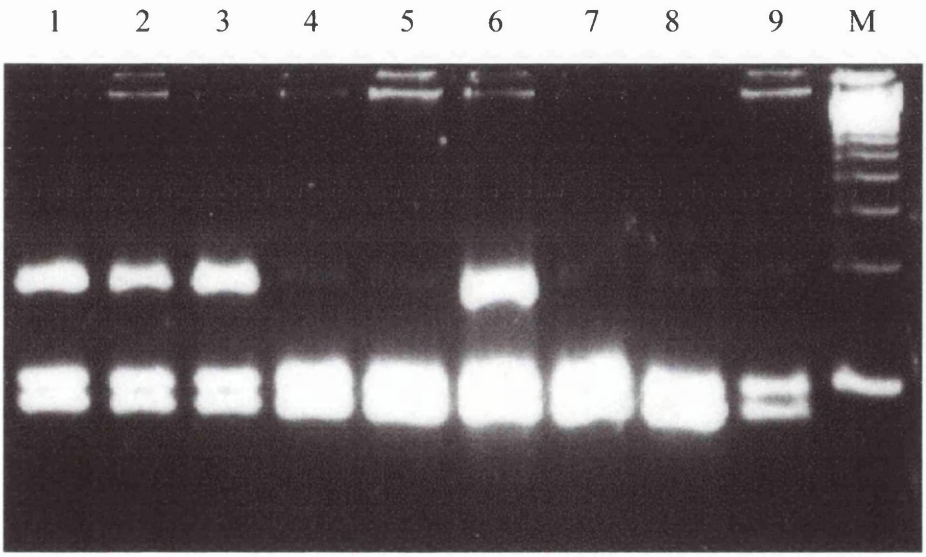
The frequencies of the *IL1A –889* and *IL1B +3953* genotypes and alleles in GEOP patients and controls are presented in Tables A1.4 and A1.5. Data for smokers is shown separately from non-smokers. The *IL1A2* carriage rate for the control population was 57.1%. This is higher than other published reports where carriage rates of 48.5% and 45.7% were found (McDowell *et al.*, 1995; Jouvenne *et al.*, 1999). The *IL1B +3953* genotype and allele frequencies were similar to those previously published (Pociot *et al.*, 1992; Bioque *et al.*, 1995; Hacker *et al.*, 1999). No statistically significant differences were found between patients and controls for any of the genotype or allele frequencies investigated ($p = 1.0$). When smoking status was included in the analysis as

Figure A1.2 4.0% agarose gel showing digested *IL1A* products.



Lanes 1, 3-6 and 8 show fragments of approximately 99- and 83 bp (heterozygous). Lane 2 shows a fragment of 83 bp resulting from complete digestion (homozygous for allele 1). Lane 7 shows undigested product of 99 bp (homozygous for allele 2). M = 100 bp marker. The expected 16 bp fragment is not visible on the gel due to its small size.

Figure A1.3 4.0% agarose gel showing digested *IL1B* products.



Lanes 1-3 and 6 show fragments of approximately 182-, 97- and 85 bp (heterozygous). Lane 4, 5 and 7-9 show fragments of 97- and 85 bp resulting from complete digestion (homozygous for allele 1). None of the samples shown are homozygous for allele 2, for which a fragment of 182 bp would be visible. M = 100 bp marker. The expected 12 bp fragment is not visible on the gel due to its small size.

Table A1.4 Frequency of *IL1A – 889* genotype and alleles in GEOP patients and controls

Genotype	Patients n (%)			Controls n (%)		
	27 [S]	29 [NS]	56 [T]	16 [S]	40 [NS]	56 [T]
1/1	12 (44.4)	12 (41.4)	24 (42.9)	7 (43.7)	17 (42.5)	24 (42.9)
2/1	15 (55.6)	11 (37.9)	26 (46.4)	8 (50)	17 (42.5)	25 (44.6)
2/2	0 (0)	6 (20.7)	6 (10.7)	1 (6.3)	6 (15)	7 (12.5)
Alleles	54	58	112	32	80	112
1	39 (72.2)	35 (60.3)	74 (66.1)	22 (68.8)	51 (63.75)	73 (65.2)
2	15 (27.8)	23 (39.7)	38 (33.9)	10 (31.2)	29 (36.25)	39 (34.8)

Table A1.5 Frequency of *IL1B + 3953* genotype and alleles in GEOP patients and controls

Genotype	Patients n (%)			Controls n (%)		
	27 [S]	29 [NS]	56 [T]	16 [S]	40 [NS]	56 [T]
1/1	14 (51.9)	14 (48.3)	28 (50)	10 (62.5)	18 (45)	28 (50)
2/1	11 (40.7)	10 (34.5)	21 (37.5)	4 (25)	17 (42.5)	21 (37.5)
2/2	2 (7.4)	5 (17.2)	7 (12.5)	2 (12.5)	5 (12.5)	7 (12.5)
Alleles	54	58	112	32	80	112
1	39 (72.2)	38 (65.5)	77 (68.8)	24 (75)	53 (66.2)	77 (68.8)
2	15 (27.8)	20 (34.5)	35 (31.2)	8 (25)	27 (33.8)	35 (31.2)

S = smokers, NS = non-smokers, T = total, n = number in each group.

a covariate there was still no significant difference between the patient and control groups ($p \geq 0.5$).

The composite genotype (*IL1A2/ IL1B2*) described by Kornman *et al.* (1997) was also investigated in this group of GEOP patients (Table A1.6). No significant differences were found between patients and controls whether smoking was included as a covariate or not ($p \geq 0.51$).

A1.4 Discussion

No association was found between GEOP and either *IL1A –889* or *IL1B +3953* in this study of north European Caucasians. The association previously found by Kornman *et al.* (*IL1A2/IL1B2*) in AP patients only occurred in non-smokers (Kornman *et al.*, 1997). Those non-smokers who were positive for the composite genotype had the same risk of disease as smokers who were either genotype-positive or genotype-negative. Smoking has been identified as the major environmental risk factor associated with increased incidence and severity of periodontitis (Grossi *et al.*, 1994; 1995; Page and Beck, 1997). In the study of Kornman *et al.* (1997) there were only 18 patients in the severe non-smoking group and although the odds ratio between severe versus mild AP was large (18.90), the confidence intervals were very wide (1.04-343.05). It is possible that this finding occurred by chance following multiple comparisons. In addition to the polymorphisms already mentioned, *IL1B –511* (di Giovine *et al.*, 1992), *IL1RN VNTR* (intron 2) (Tarlow *et al.*, 1993) and *TNFA* (tumour necrosis factor alpha*)-308* (Wilson *et al.*, 1992) were also investigated. No associations were found between AP and any of these alleles.

Another recent report of a population of Caucasian AP patients, found an association between allele 2 of the *IL1B +3953* gene and advanced periodontitis. This genotype occurred more frequently in patients with advanced disease, when compared to patients with either mild or moderate disease (Gore *et al.*, 1998). However, there was no significant difference between the advanced group and the healthy control group with regard to this allele. No associations were found between the *IL1A -889* gene and any of the disease categories or between patients and healthy controls. Furthermore,

Table A1.6 Frequency of composite *IL1A2 /IL1B2* genotype in patients and controls

Genotype	Patients n (%)			Controls n (%)		
	27 [S]	29 [NS]	56 [T]	16 [S]	40 [NS]	56 [T]
+	12 (44.4)	14 (48.3)	26 (46.4)	5 (31.2)	21 (52.5)	26 (46.4)
−	15 (55.6)	15 (51.7)	30 (53.6)	11 (68.8)	19 (47.5)	30 (53.6)

an investigation of the composite genotype (Kornman *et al.*, 1997) demonstrated a very significant association (p = 0.002, 2-tail Fisher's exact test) between this genotype and both patient and control groups. These findings are difficult to interpret but do appear to bring into question the results of the earlier study (Kornman *et al.*, 1997). However, the findings of the more recent study should also be treated with caution, as the numbers of individuals in the different categories of disease severity were small (n = 10 for mild, 10 for moderate, 12 for severe). Because of the small size of the groups, no analysis of the contribution of smoking as a confounding variable could be carried out (Gore *et al.*, 1998).

A recent study in North America found very significant evidence of linkage disequilibrium between allele one of the *IL1A* gene and allele one of the *IL1B* gene and GEOP (Diehl *et al.*, 1999). A number of multiplex families were used to investigate these candidate genes in EOP. In this report, both the transmission disequilibrium test (TDT) and the affected sib pair design were used to examine the association between the two *IL1* polymorphisms. The TDT test compares the number of transmissions of specific alleles from heterozygous parents to affected offspring. For GEOP subjects allele one of the *IL1A –889* polymorphism was transmitted significantly more frequently than allele two (p = 0.0065). There were seventeen transmissions of allele one of the *IL1B +3953* gene and only two transmissions of allele two (p = 0.0005). Similar but non-significant trends were found for LEOP. Associations were observed in both smokers and non-smokers. The homozygous (1,1) *IL1B +3953* genotype was also transmitted significantly more often to GEOP affected individuals than to unaffected subjects (p = 0.014). These findings were consistent in both African Americans and Caucasians, although the numbers in some categories were small. Further analyses revealed that the transmission disequilibrium is more strongly associated with the *IL1B +3953* polymorphism. These results provide evidence that the *IL1B +3953* polymorphism (allele one) may be in linkage disequilbrium with one EOP gene. Results from the affected sib pair analysis were less compelling. The finding of an association between allele one of *IL1A –889* and *IL1B +3953* and GEOP (Diehl *et al.*, 1999) is diametrically opposed to the increased frequency of the combined *IL1A2/IL1B2* reported previously in severe AP patients (Kornman *et al.*, 1997). This observation may be due to differences between diagnostic groups. Another possibility is that both reported associations are false positives due to chance

deviations in the populations studied. The study reported here has failed to find an association with any allele or combination of alleles of these polymorphisms and GEOP. However, using the TDT to investigate associations between candidate genes and complex multifactorial diseases such as periodontitis is much more likely to uncover genetic markers. This method which uses family data eliminates the problem of matching cases and controls. In addition, the possibility of aggregation in families being due to a shared family environment, such as diet, oral hygiene or transmission of virulent strains of bacteria, is eliminated by the TDT.

As previously mentioned the *IL1A2* allele carriage rate was higher in the control population presented here than in other published reports (McDowell *et al.*, 1995; Jouvenne *et al.*, 1999). This may be due to variation between different populations. It is also possible that the high level of oral hygiene of the control population used in this study may have masked any genetic predisposition to periodontitis. However, since the frequency of GEOP in the general population is in the region of 0.3% (Oliver *et al.*, 1998) it seems unlikely that the selection of the control population would have affected the results.

Two research groups have recently investigated the prognostic value of the composite *IL1* genotype (Kornman *et al.*, 1997) in AP patients (McGuire and Nunn, 1999; Ehmke *et al.*, 1999). The first study assessed the effectiveness of clinical parameters and *IL1* genotype in predicting prognosis and tooth survival in 42 Caucasian patients on maintenance therapy for 14 years (McGuire and Nunn, 1999). The results showed that the *IL1* haplotype increased the risk of tooth loss by 2.7-fold and heavy smoking (> 40 pack years) by 2.9-fold. The combined effect of genotype and heavy smoking increased the risk of tooth loss by 7.7-fold. These findings should be treated with caution since analysis was performed on a site rather than a patient basis. Of the 47 teeth lost because of periodontal disease, 27 were in genotype-positive individuals. It is possible that most of these teeth were extracted from one or two patients.

A subsequent study assessed the prognostic value of the *IL1* genotype on the progression of AP following non-surgical treatment (Ehmke *et al.*, 1999). Of 33 patients tested, 16 were genotype-positive. Following two years of maintenance care, no differences in the survival rate of teeth nor in sites exhibiting probing attachment loss of 2 mm or more were detected between subjects who carried the genotype and

those who did not. Further research is required in order to discover whether this genotype has a role to play in determining the outcome of the disease process in periodontitis.

In conclusion, no association was found between GEOP and the *IL1* polymorphisms investigated in the population presented here. The findings of this study and the other European study (Ehmke *et al.*, 1999) bring into doubt the usefulness of these candidate genes as markers of susceptibility to periodontitis. Alternatively *IL1A –889* and *IL1B +3953* may play only a small part in determining the level of the immune response in EOP. Therefore, investigating candidate genes such as these in moderately sized groups of unrelated patients and controls may not uncover associations which do in fact exist.

Appendix 2 DNA Analysis of *IL1* Polymorphisms in a Large Caucasian Family with Generalised Early Onset Periodontitis

A2.1 Introduction

The results of disequilibrium analysis of the *IL1A –889* (McDowell *et al.*, 1995) and *IL1B +3953* (Pociot *et al.*, 1992) polymorphisms in a Caucasian population of unrelated patients with GEOP and a control group was presented in Appendix One. No associations were found between any allele individually or any combination of alleles (Hodge *et al.*, 1999). It has been suggested that associations between candidate genes and diseases may be masked in large unrelated populations because cases and controls are not genetically homogeneous.

A study of a large Scottish Caucasian family with a high proportion of individuals affected by GEOP was presented in Chapter Five. The kindred was found to be suitable for linkage analysis. It is suggested that investigating the *IL1A –889* and *IL1B +3953* polymorphisms in this large family with GEOP may uncover associations which were hidden in the earlier study of unrelated patients and controls (Hodge *et al.*, 1999). The aim of this study was to test this hypothesis.

A2.2 Materials and Methods

A2.2.1 The Large Kindred

The method of diagnosis of the proband and relatives of the proband has been described in sections 5.2.1, 5.2.2 and 2.3.1. Three categories were defined according to reliability of diagnosis of EOP. The extent and severity of disease for age were also taken into consideration. The categories were "definitely", "probably" and "possibly" affected. Figure A2.1 briefly summarises the process of diagnosis. A pedigree of the large family is shown in Figure A2.2.

Figure A2.1 Flow diagram for diagnosis of EOP relatives

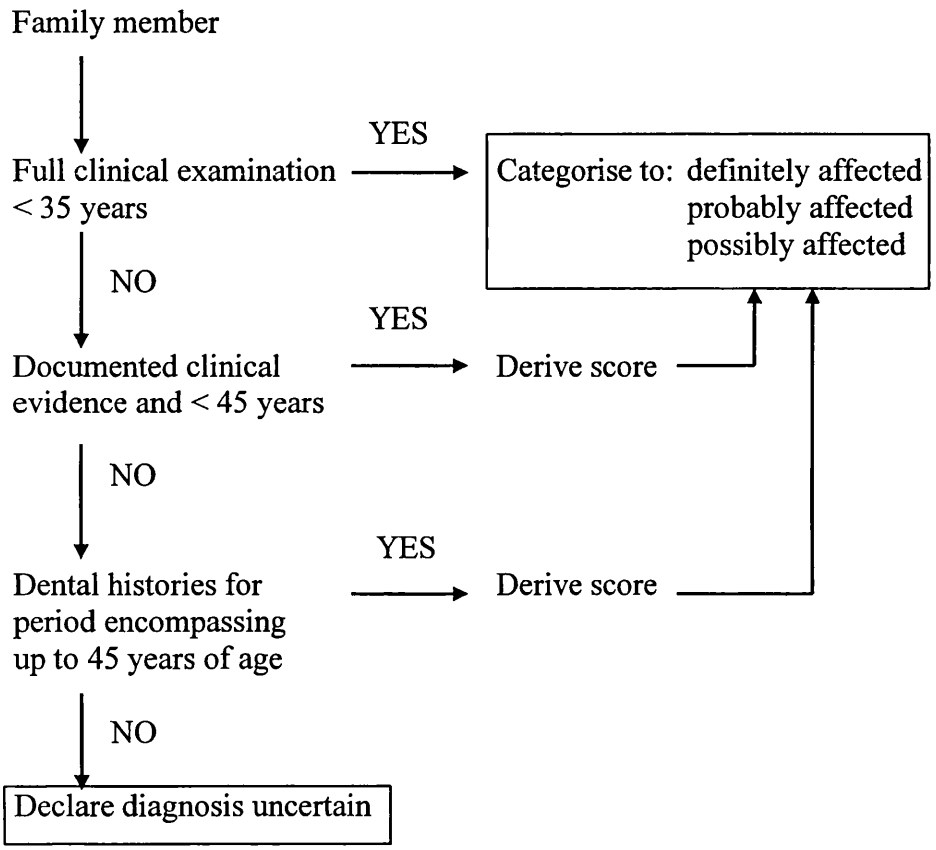
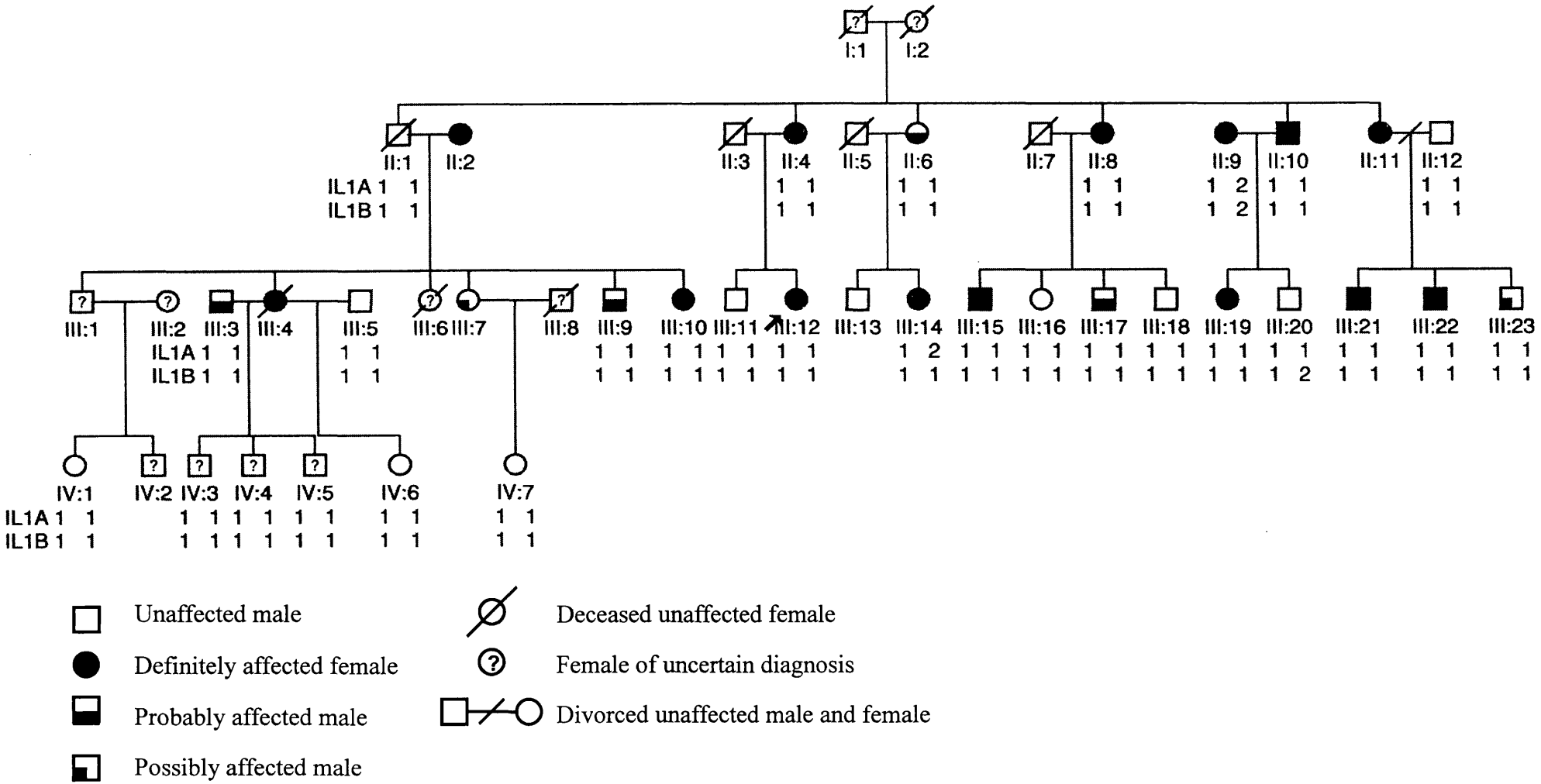


Figure A2.2 Pedigree of the large family to show *IL1* genotypes.



A2.2.2 DNA Purification

DNA separation was performed as described in section 3.2.3. DNA integrity was checked and DNA quantitated using agarose gel electrophoresis as previously described (sections 2.3.5 and 3.2.3).

A2.2.3 Screening of Relatives for the *IL1A* and *IL1B* Polymorphisms

The screening of relatives for the *IL1A –889* and *IL1B +3953* polymorphisms was previously described in section A1.2.4.

A2.2.4 Statistical Analysis

The program Mlink, part of the Fastlink version of the LINKAGE package (Lathrop *et al.*, 1984; Lathrop and Lalouel, 1984; Lathrop *et al.*, 1986), was used to investigate the presence of linkage between the *IL1* polymorphisms and a GEOP susceptibility gene in the large family. Linkage is the association within families of two or more non-allelic genes resulting from their proximity on the same chromosome. This program calculates the lod score for each polymorphism for a set of recombination fractions. Recombination is the formation of new combinations of linked genes by crossing over between loci. The recombination fraction (θ) is the fraction of meiotic events that show a recombination between two loci. The lod score is a base 10 logarithm of the odds favouring linkage. A lod score of +3 (1000:1 odds) is taken as proof of linkage; a score of -2 (100:1 odds against) indicates no linkage.

In order to carry out linkage analysis of these candidate genes as possible markers for EOP the allele frequencies of the control population presented in Table A1.4 and A1.5 were required. The model assumed 75% penetrance up to 25 years of age and 97% thereafter. Penetrance is the observable expression of the mutant gene. A phenocopy rate of 3% was used in the analysis. The population prevalence of GEOP was set at 0.3% (Oliver *et al.*, 1998). This gave an allele frequency of 0.15%.

A2.3 Results

A2.3.1 Digested PCR Products

Digestion of the *IL1A* and *IL1B* PCR products has been previously described in section A1.3.3. The results of the genotyping of these polymorphisms for the large family are illustrated in Figure A2.2. Almost all of the relatives who had blood samples available for analysis carried the homozygous 1/1 genotype for both polymorphisms. Two family members (II:9 and III:14) were heterozygous for *IL1A –899* and two (II:9 and III:20) were heterozygous for *IL1B +3953*. Because of the high degree of homozygosity for alleles of *IL1A -899* and *IL1B +3953* in this family it was not informative for linkage

A2.3.2 Statistical Analysis

The results of the linkage analysis are presented in Table A2.1. It can be seen that the data provide very little information about linkage to either marker. Since nearly all members typed are homozygous for allele one of *IL1A* and *IL1B* it is not possible to detect whether recombination has taken place. The affected members are unlikely to be homozygous for the disease, yet they have the double *A1.1/B1.1* haplotype. Considering the high frequency of allele one of *IL1A* and *IL1B* in the general population, and the closeness of the *IL1A* and *IL1B* markers, it is not surprising that the *A1B1/A1B1* genotype should appear in incoming parents as well. Where it is detectable, there is evidence of recombination either between the disease gene and *IL1A* or between *IL1A* and *IL1B*.

A2.4 Discussion

No association was found between GEOP and either *IL1A –889* or *IL1B +3953* in this family study of north European Caucasians. The results of the linkage analysis presented in Table A2.1 indicate that this family is uninformative for linkage of these *IL1* genes. A lod score of +3 is taken as proof of linkage and a score of -2 indicates no linkage. It can be seen from the data presented in Table A2.1 that the lod scores range from 0.1 to 0.01 for the *IL1* polymorphisms when tested over a range of recombination fractions from 0.0 to 0.4.

Table A2.1 Lod scores for two point analyses of the *IL1A –889* and *IL1B +3953* polymorphisms and a mutant EOP gene in the large pedigree

Recombination fraction	*IL1A -889*	*IL1B +3953*
0.0	0.08	0.10
0.01	0.08	0.09
0.05	0.07	0.08
0.1	0.06	0.07
0.2	0.04	0.04
0.3	0.02	0.02
0.4	0.01	0.01

The finding of an association between allele one of *IL1A –889* and *IL1B +3953* and GEOP in a study of 35 multiplex families in North America has been discussed in section A1.4 (Diehl *et al.*, 1999). It is possible that the present study failed to find an association because of the heterogeneous nature of EOP. Different families may carry different susceptibility genes. In addition the lack of heterozygosity among generation II of the large family precluded the use of the TDT. There was a high prevalence of the homozygous (1/1) genotype for *IL1A –889* and *IL1B +3953* among both affected and unaffected family members (Figure A2.2).

In conclusion, the analysis of this large kindred failed to either confirm or reject an association between the *IL1A –889* and *IL1B +3953* polymorphisms and GEOP. These results together with the findings of studies discussed previously (section A1.4) bring into doubt the usefulness of these *IL1* polymorphisms as markers of susceptibility to periodontitis at present. If they do play a small role in determining the level of the immune response, they may in the future be found to form part of an extended haplotype, which underlies a predisposition to this disease.

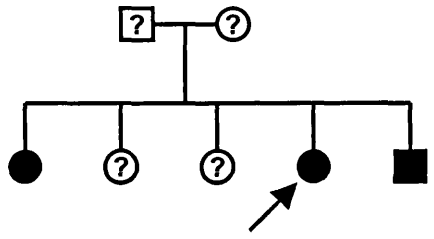
Appendix 3 Pedigrees of Additional Families with Early Onset Peridontitis

A3.1 Introduction

The following appendix contains pedigrees of nuclear and multiplex families which were identified in the search for the large family presented in Chapter Five. The same system of diagnosis has been used for these pedigrees as is presented in section 5.2.1, 5.2.2 and 2.3.1.

Figure A3.1 Pedigrees of nuclear and multiplex families with EOP

1.



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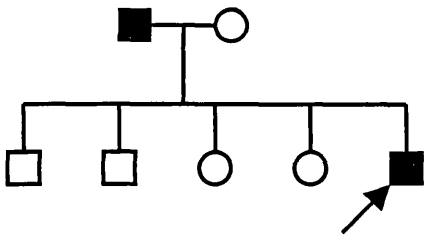

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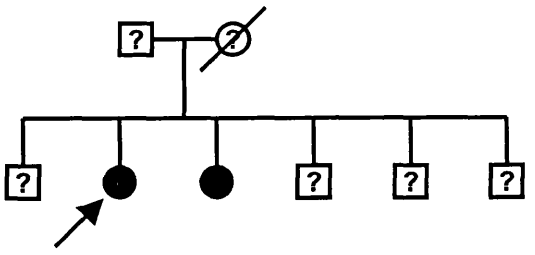
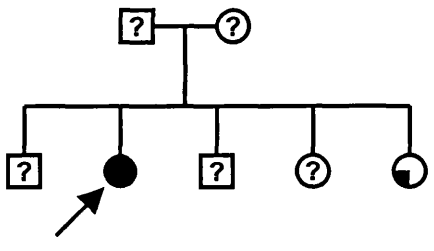
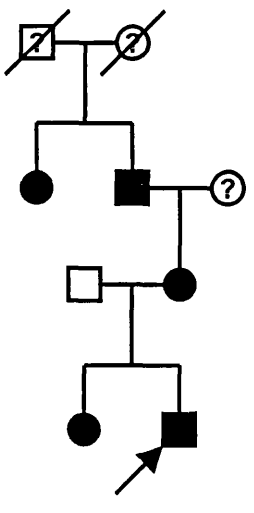


4.



5.



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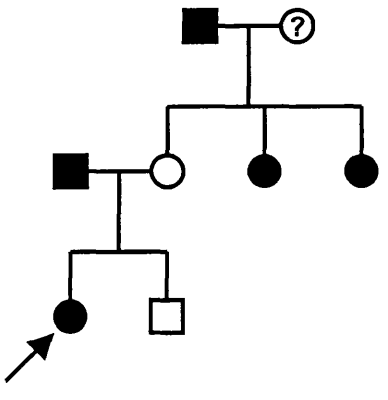
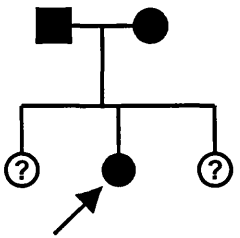


Figure A3.1 (continued)

7.



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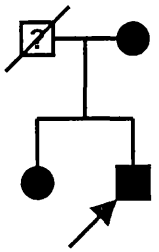
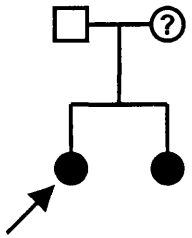


9.



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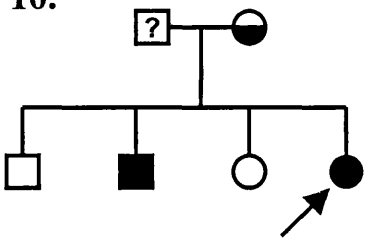

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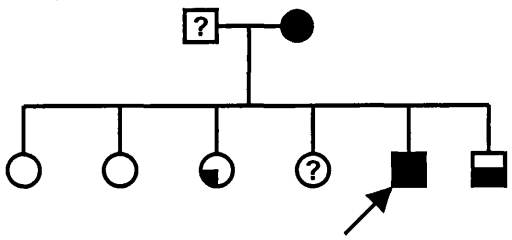

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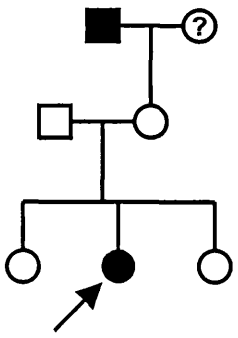

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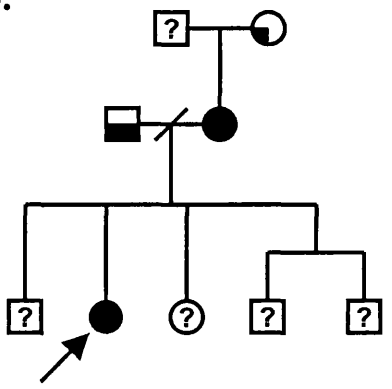

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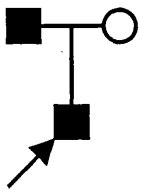

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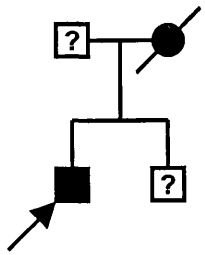

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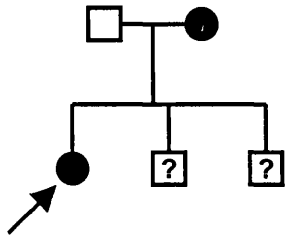

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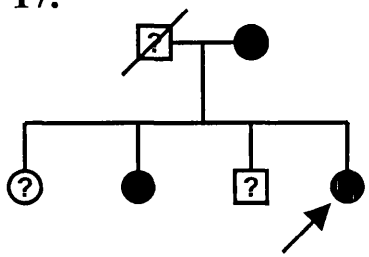

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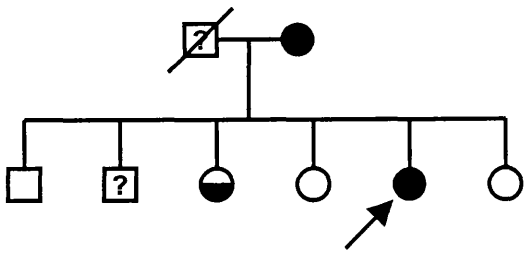

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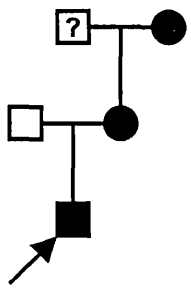

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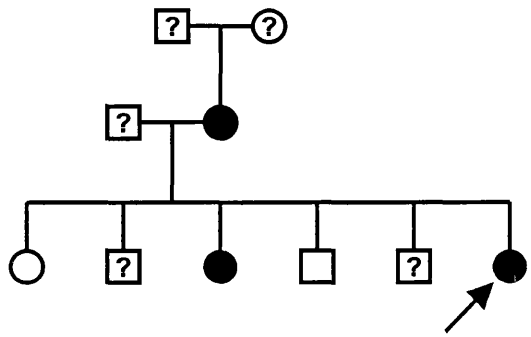

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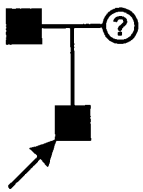

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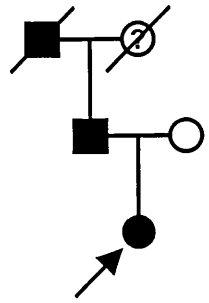

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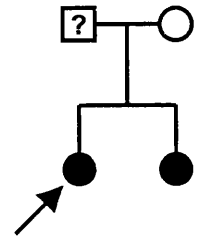

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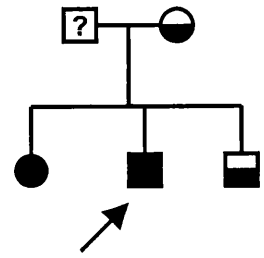

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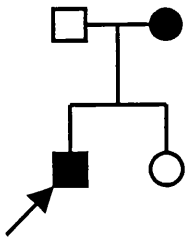

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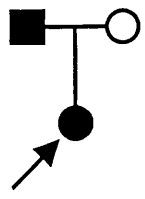

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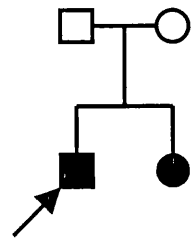

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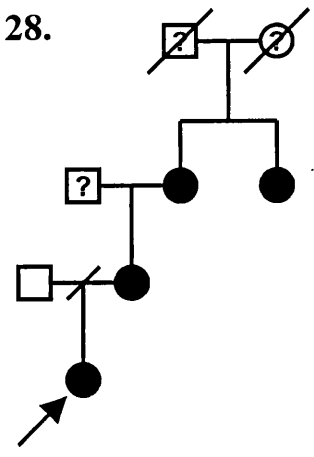

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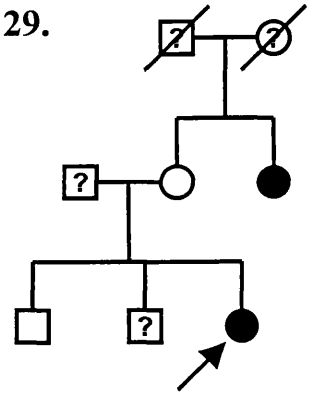

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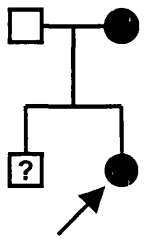

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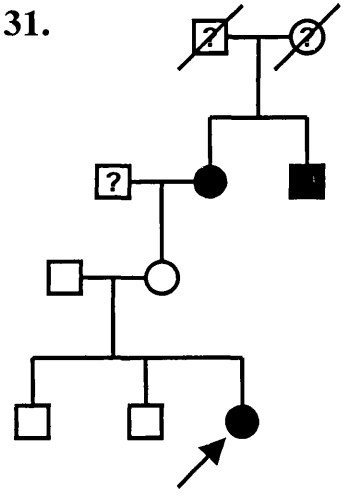

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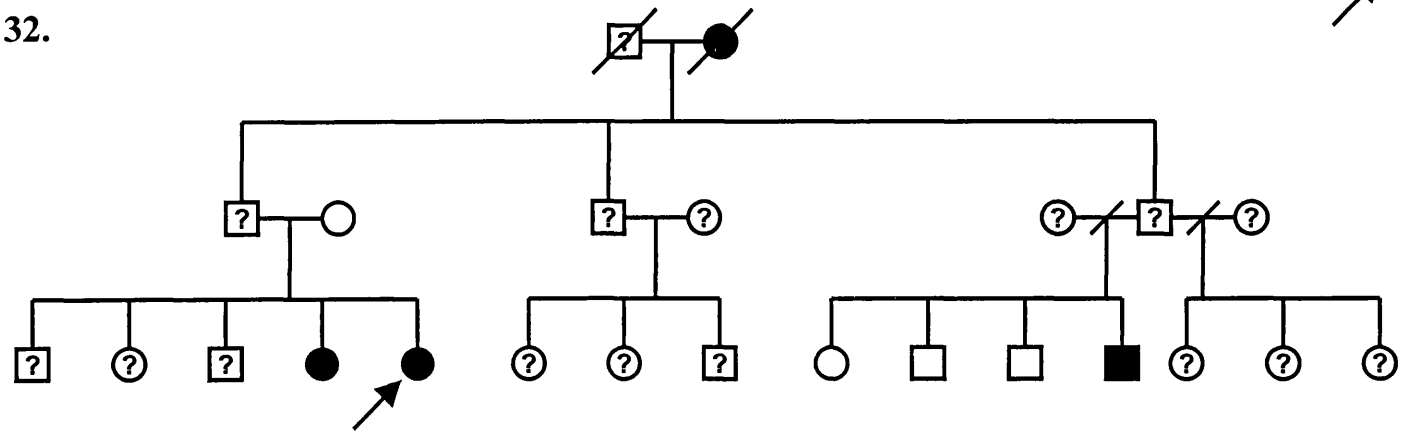

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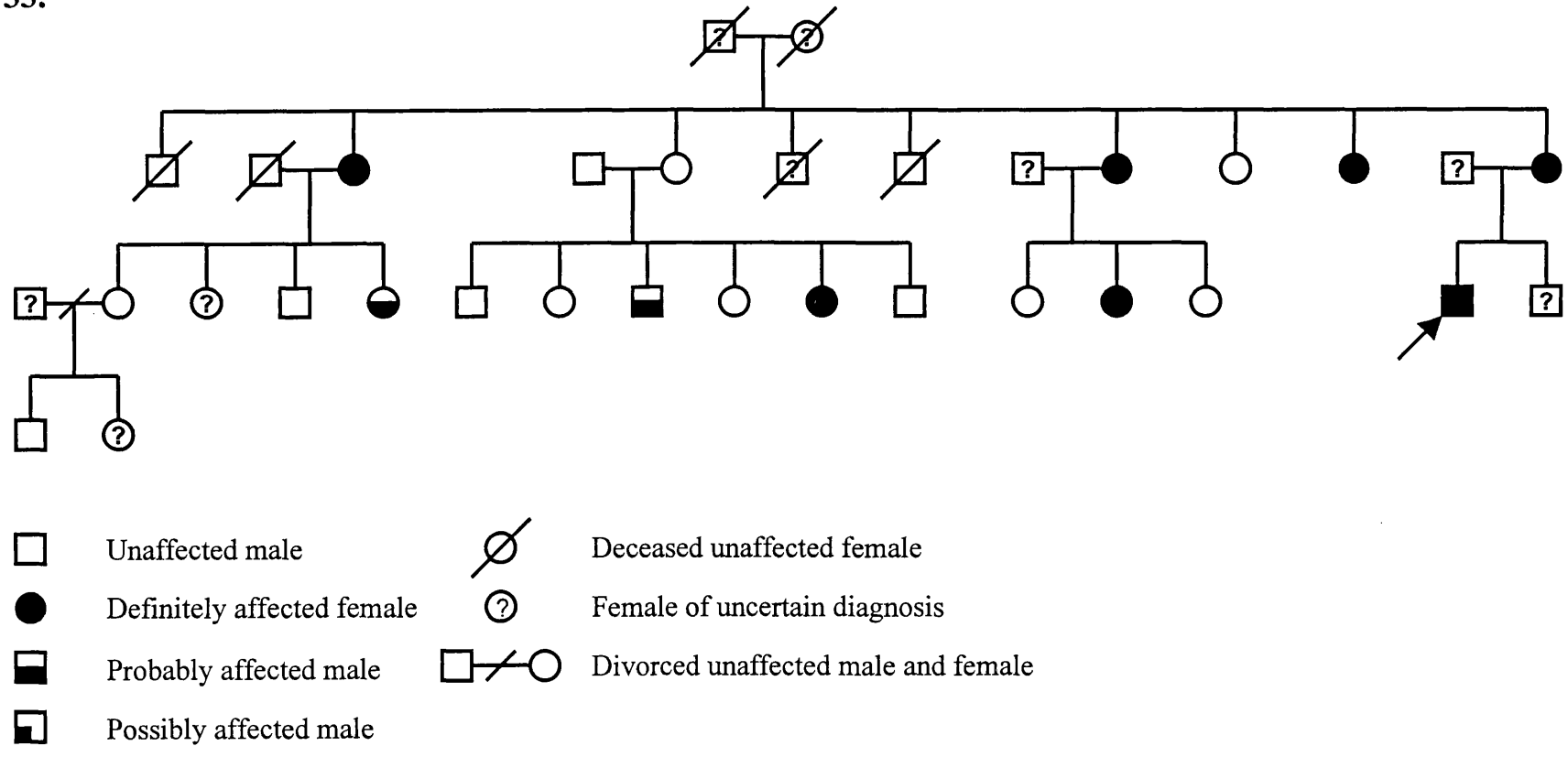

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List of Publications

The following papers are directly related to the work presented in this thesis:

Hodge, P.J., Riggio, M.P. and Kinane, D.F. (1999) No association with HLA-DQB1 in European Caucasians with early-onset periodontitis. *Tissue Antigens*, **53**, 205-207.

Kinane, D.F., Hodge, P., Eskdale, J., Ellis, R., Gallagher, G. (1999) Analysis of genetic polymorphisms at the interleukin-10 and tumour necrosis factor loci in early-onset periodontitis. *Journal of Periodontal Research*, **34**, 1-8.

Hodge, P.J. and Michalowicz, B.S. (2000) Genetic predisposition to periodontitis in children and young adults. *Periodontology 2000* (in press).

Hodge, P.J., Teague, P.W., Wright, A.F. and Kinane, D.F. (2000) Clinical and genetic analysis of a large north European Caucasian family affected by early-onset periodontitis. *Journal of Dental Research* (submitted).

Hodge, P.J., Riggio, M.P. and Kinane, D.F. No association with *IL1* genotypes in European Caucasians with generalised early-onset periodontitis. *Journal of Periodontology* (to be submitted for publication December 1999).

The following papers are indirectly related to the work presented in this thesis:

Lappin, D.F., Koulouri, O., Radvar, M., Hodge, P. and Kinane, D.F. (1999) Relative proportions of mononuclear cell types in periodontal lesions analyzed by immunohistochemistry. *Journal of Clinical Periodontology*, **26**, 183-189.

Darby, I.B., Hodge, P.J., Riggio, M.P., Kinane, D.F. (2000) Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. *Journal of Clinical Periodontology*, **27**, (6) (in press).

Mooney, J., Hodge, P.J. and Kinane, D.F. Humoral immune response in early-onset periodontitis: influence of smoking. *Journal of Periodontal Research* (to be submitted for publication December 1999).

Abstract presentations

The following abstracts are directly related to the work presented in this thesis:

Hodge, P.J., Teague, P.W., Wright, A.F and Kinane, D.F. (1998) Clinical and genetic analysis of a large family with early-onset periodontitis. *Journal of Dental Research,* **77** (special issue), 647.

Hodge, P.J., Riggio, M.P. and Kinane, D.F. (1999) IL-1 genotypes in European Caucasians with generalised early-onset periodontitis. *Journal of Dental Research,* **78** (special issue), 381.

The following abstracts are indirectly related to the work presented in this thesis:

Hodge, P., Mooney, J. and Kinane, D.F. (1998) Humoral immune response in early-onset periodontitis: influence of smoking. *Journal of Dental Research,* **77** (special issue), 1032.

Darby, I.B., Hodge, P.J., Riggio, M.P. and Kinane, D.F. (1999) Comparative microflora of adult and early-onset periodontitis. *Journal of Dental Research,* **78** (special issue), 295.