THE CHARACTERISATION OF MACROPHAGE FUNCTIONS
IN UNTREATED ADULT PERIODONTITIS

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This thesis is dedicated to Katarina and Ben
STATEMENT OF AUTHORSHIP

The experimental work described in this thesis was performed by the candidate except where stated otherwise. This work has not been submitted in whole or in part for any other degree.

Cheryl J. Stapple

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PUBLICATIONS

The following papers contain results described in this thesis.

**Primary studies:**


**Supporting studies as a co-author, not described in detail in data chapters:**

Kumar RK, Chapple CC, Hunter N. Improved double immunofluorescence for confocal laser scanning microscopy. *J Histochem Cytochem* 1999 ; **47**: 1213-8


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LIST OF ABBREVIATIONS

%  percentage
±  plus or minus
≥  greater than or equal to
≤  less than or equal to
°C  degrees Celsius
µm  micrometre
<  less than
25F9  resident macrophage antibody
27E10  acute stage inflammatory macrophage antibody
AP  acid phosphatase
APC  antigen presenting cell
B cell  B lymphocyte
bFGF  basic fibroblast growth factor
BSA  bovine serum albumin
CD  cluster of differentiation
CD68  pan macrophage antibody
CR  crest
CT  connective tissue
DAB  diaminobenzidine
DC  dendritic cell
ECM  extracellular matrix
et al.  et alii (Latin) = and others
FCS  foetal calf serum
GCF  gingival creviccular fluid
GM-CSF  granulocyte macrophage-colony stimulating factor
h  hour
HLA  human-leukocyte-associated
HS  horse serum
ICAM-1  intercellular adhesion molecule-1
ICI  inflammatory cell infiltrate
IFN  interferon
IL  interleukin
JE  junctional epithelium
LC  Langerhans cell
LDL  low density lipoprotein
LPS  lipopolysaccharide
MAC3  Ber-MAC3 a high specificity macrophage antibody
MCP  monocyte chemoattractant protein
M-CSF  macrophage-colony stimulating factor
MHC  major histocompatibility complex
Abbreviations are used for frequently mentioned terms. Normal chemical nomenclature is used in this thesis.

Throughout this thesis the generic term “macrophage” is used. When it is necessary to distinguish these cells the terms “monocyte-derived macrophage”, “inflammatory macrophage”, “resident macrophage” or “histiocyte” are used.

The terms “periodontitis”, “adult-type periodontitis” and “adult periodontitis” are interchangeable for the purposes of this thesis.

In this thesis the terms “subpopulation” and “subset” are interchangeable and refer to a group of macrophages displaying the same phenotypic or functional marker. Furthermore, it is recognised that the marker expressed by the macrophage reflects that cell’s function or phenotype at one point in time.

The monoclonal antibody Ber-MAC3 is referred to as MAC3 in Chapters 5 through to 8.
SUMMARY

Macrophages play a critical role in many chronic inflammatory diseases. The pleiotrophic functional capacity of these cells includes phagocytosis and killing of opsonised microorganisms, antigen presentation to T cells, cytotoxicity and the secretion of potent angiogenic growth factors, of central importance in the maintenance or restoration of tissue homeostasis.

Although the pathology of the chronic inflammatory disease, periodontitis, has been studied extensively there is a paucity of data relating to the role of macrophages. Recent studies of the untreated advanced periodontitis lesion have revealed extensive vascular pathology, including alterations in blood vessel morphology with thickening of the basement membrane, the accumulation of fragments of vascular basement membrane, persistence of foci of degenerate plasma cells and a restricted capacity to develop reparative granulation tissue. In relation to these pathological features, the distribution and functional status of macrophages assumes prime importance.

Macrophage populations in untreated advanced periodontitis biopsies were compared with those in biopsies of clinically healthy, minimally inflamed, gingival tissue. The immunohistochemical investigation used high specificity monoclonal antibodies including a pan-macrophage marker and probes for acute inflammatory, resident histiocytic, and reparative macrophage phenotypes. Results indicated that advanced periodontitis and minimally inflamed tissues displayed similar distribution patterns and numbers for the macrophage phenotypic markers. Regionally-specific differences in the populations occurred, however.

Further studies investigated macrophage expression of the functional activation markers MHC class II for antigen presentation, and acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP) for lysosomal enzyme activity. In the advanced periodontitis lesion there was little evidence of macrophage activation for the expression of HLA-DR and TRAP, although strong expression of HLA-DR was observed in association with blood vessels. Macrophages expressing AP showed a distinct regional distribution; however, this was not associated with foci of degenerate plasma cells.
These data support the hypothesis that macrophages do not express appropriate activation for key functions in the untreated lesion of periodontitis. It is postulated that the apparent failure of recruitment and activation of macrophages may in part be both a cause and a consequence of the unusual pathological features of this disease.

Although periodontitis is a chronic inflammatory disease of the highly vascularised supporting tissues of the teeth, little is known about the vascular changes in untreated advanced periodontitis. Observations of vascular features of the pathology were investigated and defined, forming the basis for a study of two angiogenic growth factors in periodontitis. In the connective tissue subjacent to the altered epithelium lining the periodontal pocket, there was a significant increase in the numerical density of vascular profiles, primarily accounted for by vessels ≥25µm in diameter. In addition, vascular basement membranes were thickened and there was regional accumulation of non-vascular basement membrane remnants. The co-localisation of type IV collagen and laminin and the discrete nature of these structures was confirmed, using 3-D reconstruction.

The distribution of two major angiogenic growth factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), in untreated periodontitis was studied using immunohistochemistry. Basic fibroblast growth factor was not expressed by macrophages. Although bFGF was consistently associated with blood vessels, there was no regional variation in its distribution. In contrast, there was marked regional variation in the intensity of immunostaining for VEGF, with significantly reduced staining of the pocket epithelium. Few macrophages of the Ber-MAC3 phenotype expressed VEGF, as determined by dual immunofluorescence and confocal microscopy. It is considered that the changes in the vascularity of the periodontal connective tissues in untreated advanced periodontitis are, in part, a consequence of altered expression of angiogenic activity by the epithelium and limited expression of angiogenic growth factors by macrophages.

Macrophage anergy and vascular disruption observed in the described studies indicate that tissue defence, maintenance and repair are compromised in the untreated lesion of advanced periodontitis.
CHAPTER 1
INTRODUCTION

THE PROBLEM

Macrophages play a critical role in many chronic inflammatory diseases. This remarkable cell has pleiotrophic functional capacity in chronic inflammatory diseases that can include phagocytosis and killing of opsonised microorganisms, antigen presentation to T cells, cytotoxicity and the secretion of over one hundred products, including potent growth factors important for angiogenic activities.

Although the pathology of the chronic inflammatory disease, periodontitis, has been studied extensively there is a paucity of data relating to the role of macrophages in this disease process.

Recent studies of the untreated advanced periodontitis lesion have revealed extensive vascular pathology, including: alterations in blood vessel morphology with thickening of the basement membrane and the accumulation of fragments of basement membrane; marked disturbances in the epithelium lining the periodontal pocket; persistence of foci of degenerate plasma cells; and a restricted capacity to develop reparative granulation tissue. In relation to these pathological features, the distribution and functional status of macrophages assumes importance.

AIM OF THE STUDY

Thus, the aim of this study was to examine the distribution of macrophages and activation for key functions in relation to observed pathological features in the untreated lesion of chronic periodontitis. The hypothesis to be tested was that macrophages do not express appropriate activation for key functions in the untreated lesion of periodontitis.

RESEARCH APPROACH

To achieve this aim a general overview of important aspects of macrophage heterogeneity, activation and functions is provided in Chapter 2. In Chapter 3, the diversity of the macrophage is reviewed and key properties relevant to chronic inflammatory diseases are described. Also, the tools available for studying the
macrophage *in situ* are reviewed. Chapter 4 describes the chronic inflammatory disease, periodontitis. The advances made and problems involved in understanding this disease, as well as knowledge concerning macrophages in periodontitis, are discussed. Chapters 5 to 8 are based extensively on the published studies “Failure of macrophage activation in destructive periodontal disease.” and “Vascular remodelling in chronic inflammatory periodontal disease.” To elaborate, the study described in Chapter 5 examines the distribution of different phenotypic macrophage populations in periodontitis whereas Chapter 6 describes key markers of functional activation for digestive capacity and antigen presentation in macrophages within the advanced periodontitis lesion. In Chapter 7, observations of vascular features of the pathology are investigated and defined, forming the basis for a study described in Chapter 8 that investigates the source of two angiogenic growth factors in periodontitis. Each research chapter contains a discussion section where the significance of the data is considered. The final chapter examines the achievements of these studies while also identifying the limitations of the work. A discussion of future directions resulting from this research completes the chapter.
CHAPTER 2
A REVIEW OF THE MACROPHAGE

2.1 INTRODUCTION

"Macrophage" meaning big eater was the name proposed by Metchnikoff in 1892, to describe a large cell with phagocytic ability found in most tissues and body fluids. Macrophages are an ancient cell population. They have been the principal motile defence cells throughout phylogeny occurring in all invertebrate classes from the simplest metazoa to all vertebrates (Metchnikoff, 1891). Macrophages were the main host defence mechanism until the development of the immune system. Co-evolution of the macrophage system and the more recent specific immune system is marked by extensive integration, overlap and cooperation. Hence macrophages have acquired important functions in the regulation of humoral and cellular immune reactions as well as in the control of cell functions of various non-phagocytic cells (van Rooijen et al., 1996).

2.1.1 Mononuclear phagocyte system (MPS)

Monocytes and macrophages were originally classified as cells of the reticulo-endothelial system (RES) based on the criteria of cells sharing a common ancestry, morphology and functions (Aschoff, 1924). Subsequently, it was discovered that not all RES cells met the requirements of the classification.

In 1972 van Furth et al., proposed the Mononuclear Phagocyte System (MPS) based on accumulating data on the origin and kinetics of monocytes and macrophages under normal and pathological conditions (Volkman, 1966, 1970, van Furth and Cohn, 1968, van Furth et al., 1973).

Therefore, the MPS is composed of cell types believed to belong to the same lineage as the macrophage, such as monoblasts, promonocytes, monocytes, microglia, Kupffer cells, and osteoclasts (van Furth, 1988). Collectively these cells are referred to as mononuclear phagocytes. This term although widely used can be misleading because it over-emphasises the phagocytic function when other functions may be of similar
importance. Also some cells, such as foreign body giant cells and osteoclasts display syncytia formation.

The vast heterogeneity of the MPS raises the question of which cells are members. Monocytes, for example, have been recognized as the precursors of Langerhans cells (Strobl et al., 1998), yet the place of the family of dendritic leukocytes, comprising Langerhans cells, dendritic cells, veiled cells, and the interdigitating cells within the MPS remains ambiguous (Radzun et al., 1988, Damoiseaux et al., 1989, Cella et al., 1997, Strobl et al., 1998). Furthermore, although there are monoclonal antibodies that identify cells belonging to the MPS, there has been to date none that exclusively recognise all MPS members.

2.1.2 Macrophage origins

Macrophages originate from monocytes which in turn are derived from rapidly proliferating precursors in the bone marrow. This has been firmly established by labelling studies in rats (Volkman and Gowans 1965a, b, Spector et al., 1965) and mice (van Furth and Cohn, 1968).

Arising from pluripotent stem cells, the granulocyte-macrophage progenitor (CFU-GM) is a multipotent cell that can differentiate to macrophages, granulocytes or osteoclasts. The monoblast and promonocyte in the bone marrow give rise to monocytes, which remain in the bone marrow for 1.5 to 3 days, before entering the circulation. Monocytes, although regarded as young cells, already possess migratory, chemotactic, pinocytic and phagocytic activities as well as receptors for C3bi and IgG Fc-domains. Monocytes reside in the circulation for about 6 days (reviewed by Volkman, 1970) under normal physiological conditions before migrating into the tissues where maturation is completed and differentiation into multifunctional macrophages proceeds (Volkman and Gowans, 1965a, b, Volkman, 1970, reviewed by van Furth, 1988).

Contributing to macrophage numbers in the tissues is a small proportion, less than 5%, of DNA-synthesising macrophages, derived from recently arrived monocytes, and possessing a limited capacity for division (Blusse van Oud Alblas et al., 1983).
2.2 MACROPHAGE DIFFERENTIATION

Differentiation, the process of development along an increasingly restricted lineage, is normally an irreversible event initiated by growth factors and cytokines (Rutherford et al., 1993). Unlike studies on lymphocyte differentiation which have utilised the phenomenon of genomic reorganisation, monitoring macrophage differentiation is relatively imprecise, relying on physical or functional changes to identify developmental stages.

The concept that monocyte/macrophage differentiation takes place in stages has been supported by studies of macrophage activation (Hamilton and Adams, 1987). Also, substantial data on the diversity of macrophage differentiation within different tissues has been obtained by studying surface molecules. As monocytes differentiate and subsequently mature into macrophages, the expression of various cell-surface antigens alters; for example, new receptors appear such as the receptor for the lipopolysaccharide (LPS) binding protein-LPS complex (CD14), whereas other receptors disappear (Nibbering et al., 1987a). Significantly, as monocytes enter specific tissues or anatomical sites within a tissue they differentiate into different macrophage phenotypes in response to environmental stimuli (Nibbering et al., 1987b). As a result, macrophages from different organs and from different anatomical sites within an organ, differ from one another. For example, alveolar macrophages do not resemble pulmonary tissue macrophages and peritoneal macrophages do not resemble any other population of macrophages.

2.3 MACROPHAGE HETEROGENEITY

Macrophages exhibit extensive heterogeneity with respect to morphology, biochemistry, surface antigens, secretory products and functions. The origin of macrophage heterogeneity is however, not fully understood, and several models have been proposed. Rutherford et al., 1993, supported multiple mechanisms by which heterogeneous macrophage populations could arise. These include: clonal variation of myeloid progenitor cells; developmentally-staged expression of specific functions; differential signals experienced within diverse tissue microenvironments; and alternate haematopoietic stimulation by the macrophage-colony stimulating factor (M-CSF) and
granulocyte macrophage-colony stimulating factor (GM-CSF), produced in situ and/or supplied humorally. These potential mechanisms are presumably not mutually exclusive and each may contribute in some degree to the heterogeneity of macrophage populations (Rutherford et al., 1993). Another model proposed by Takahashi et al., (1996), postulates distinct differentiation pathways in the development of macrophage heterogeneity. This model emphasises the essential role of growth factors, namely M-CSF and GM-CFS, in the haematopoietic microenvironment from which the heterogeneous macrophage populations arise.

Macrophage heterogeneity arising from the effect of inflammatory stimuli on macrophage precursor cells is reviewed in the following chapter.

2.4 MACROPHAGE ACTIVATION

From the seminal studies of Mackaness and co-workers, macrophage activation was defined as a T cell-dependent enhanced killing action for refractory organisms, including Listeria monocytogenes (Mackaness, 1962, 1964). Later it was found that the T-cell product mediating the activation was interferon-gamma (IFN-γ) (Nathan et al., 1983). The Mackaness definition of activation was contrasted with the concept of stimulated macrophages, that is an increase in a variety of functions in response to challenge, commonly with bacterial lipopolysaccharide (LPS). The distinction between activation and stimulation has been merged over time and in accordance with this trend, macrophage activation will be used to describe enhancement of any important function of these cells. It should be stated, however, that activation by any one agonist does not lead to simultaneous enhancement of all macrophage functions. Indeed, the enhanced ability to perform one function can occur at the expense of another.

The concept of the requirement for activation prevails in almost all studies of macrophage function. The potent role of activated macrophages in angiogenesis is a good example of this (Sunderkotter, 1994).
2.5 MACROPHAGE FUNCTIONS

The multifunctional role of macrophages in host defence reactions has been established. These cells are responsible for the non-immune resistance against microorganisms and grafted cells. They act as accessory cells for the induction of cellular and humoral immune reactions against particulate antigens and they regulate functions of various non-phagocytic cells which participate in immune responses (van Rooijen et al., 1996).

Mackaness' concept of cell-mediated immunity, published in 1962 and 1964, was based on experiments using a simple murine model of infection by *Listeria monocytogenes*. These experiments laid the foundation for the analysis of immune mechanisms against infection with facultative intracellular microorganisms and have led to an array of data elucidating the mechanisms of innate resistance and acquired immunity. The role of the macrophage in these mechanisms has been established.

Under normal physiological conditions macrophages perform sentinel and housekeeping functions by removing effete cells and debris, and secreting an array of bioactive molecules important for tissue maintenance and repair.

Of particular relevance to the work in this thesis are the functions that are performed by the macrophage in chronic inflammation. In the next chapter a selection of key macrophage functions important in chronic inflammatory diseases and presumably relevant to chronic periodontitis is presented.
CHAPTER 3

THE MACROPHAGE IN CHRONIC INFLAMMATION

3.1 INTRODUCTION

Monocyte-derived macrophages participating in an inflammatory response are also referred to as inflammatory or exudative macrophages. These macrophages are a heterogeneous population of cells that differ in the stage of differentiation and function. Inflammatory macrophages are present in various exudates. A variety of specific markers e.g. peroxidase activity, may characterise these cells, and since they are derived exclusively from monocytes they share similar properties. In contrast, the resident tissue macrophage (histiocyte) is characteristically relatively quiescent immunologically, having low oxygen consumption, low levels of major histocompatibility complex (MHC) class II gene expression, and little or no cytokine secretion. Resident macrophages are, however, phagocytic, responsive to chemotactic stimuli and retain limited proliferative capacity.

3.2 MACROPHAGES IN INFLAMMATION

3.2.1 Macrophage precursor cells

Production of the macrophage lineage from bone marrow progenitors is normally controlled by M-CSF, which is constitutively produced by many cell types. During acute inflammation the number of circulating monocytes increases, and a large proportion of these cells migrate to the site of inflammation and differentiate into inflammatory macrophages. Studies in vitro indicate that factor increasing monocytopoiesis (FIM) is a long-range regulator that signals the bone marrow to increase monocyte production, by increasing mitotic activity of monoblasts and promonocytes, during an acute demand for more monocytes and inflammatory macrophages (van Furth, 1985, Annema et al., 1992). The monokine, FIM is synthesised and secreted by macrophages upon phagocytosis at the site of inflammation (Sluiter et al., 1990). Also, in response to invasive stimuli and inflammation, serum levels of M-CSF increase dramatically. GM-CSF also appears in the serum. Evidence from studies in vitro supports an indirect GM-
CSF/M-CSF interaction for enhancing numbers of circulating blood monocytes (Gilmore et al., 1995). From the large pool of macrophage progenitors able to respond to M-CSF or GM-CSF there is a considerable overlap. Differences in structures and signal transduction mechanisms of the receptors for M-CSF and GM-CSF indicate that the differentiation pathways they initiate are likely to be dissimilar (Takahashi et al., 1996).

M-CSF-derived macrophages are large and have a high phagocytic capacity. Conversely, GM-CSF-derived macrophages are more cytotoxic against tumour necrosis factor-alpha (TNF-\(\alpha\))-resistant tumour targets, express more MHC class II antigen, more efficiently kill Listeria monocytogenes, and constitutively secrete more prostaglandin (PG)E2.

3.2.2 Macrophage kinetics

Under normal steady-state conditions the population of macrophages is constant. The rate of migration of monocytes into the tissues and the small (<5%) local production of macrophages (van Furth and Cohn, 1968, van Furth, 1985) by recently arrived cells and resident tissue macrophages (histiocytes) is balanced by the rate of disappearance of macrophages from the tissues (Volkman, 1970, van Furth, 1988). The ultimate fate of macrophages is not known, however, it is widely held that macrophages die in the tissues or body cavities or migrate to other sites, such as lymph nodes, before they die.

During acute or chronic inflammation increased production of monocytes in the bone marrow results in the number of circulating monocytes increasing temporarily by two to three-fold relative to the number under normal steady-state conditions (van Furth, 1988). Labelling studies in mice have shown that at least 70% of circulating monocytes migrate to the site of acute inflammation, resulting in approximately a ten-fold increase in the numbers of macrophages in the inflammatory exudate (van Furth et al., 1973). In non-granulomatous chronic inflammation, such as periodontal diseases, macrophages at the site of the lesion are primarily bone marrow-derived monocytes. In chronic inflammation, as in acute inflammation, the contribution by local production to the number of tissue macrophages is relatively small, on average 7% (van Furth, 1992).

A hallmark of chronic inflammation is the accumulation of macrophages at the site of inflammation. The influx of monocyte-derived macrophages has been well
documented, however little is known about the mechanisms controlling persistence and clearance of macrophages from the tissues; this is important to an understanding of the pathogenesis of chronic inflammation. A recent study investigated in vivo, the fate of inflammatory macrophages during the resolution of inflammation. It was found that inflammatory macrophages, after performing scavenging activities did not die locally, by apoptosis or phagocytosis, but emigrated to draining lymph nodes where the potential for antigen presentation occurred (Bellingan et al., 1996). As this appears to be the major mechanism for macrophage clearance from the tissues, implications emerge for the initiation and amplification of immune responses against agents from the site of inflammation.

3.2.3 Macrophage recruitment

Little is known about the first signal or signals that initiate the events that ultimately lead to recruitment of macrophages and other inflammatory cells to the site of infection, tissue injury or chronic irritation. Although both mast and endothelial cells are considered important in generating early signals in the extravascular environment, it has been suggested that macrophages are well equipped for this role. Macrophages are more widely distributed throughout the tissues than mast cells and have the capacity to produce an array of cytokines and substances, such as IL-1, TNF-α and prothrombinase, capable of activating endothelial cells in the vicinity of tissue disruption (Feghali and Wright, 1997). Bacterial products, particularly LPS, have also been reported to stimulate endothelial cells (Bevilacqua, 1993), however LPS from the gram negative pathogen Porphyromonas gingivalis does not elicit E-selectin expression in human endothelial cells in vitro (Cunningham et al., 1999).

There is acceptance that the selective accumulation of leukocytes in foci of inflammation is critically mediated by receptor-ligand interactions at the luminal surface of post-capillary venules. In relation to this, microenvironmental regulation by the inflammatory milieu differentially and selectively induces the surface expression of adhesion molecules at the luminal surface of the endothelium. Leukocytes also respond by altering the affinity of integrin ligands for endothelial receptors. In addition, persistent or intermittent inflammatory reactions can be further regulated by circulating
soluble adhesion receptors that may arise from alternate splicing of the gene transcript. Transmigration of leukocytes across the vascular wall is also regulated by the nature of the available chemotactic agents (Springer, 1994).

Current models suggest that the first step in the accumulation of leukocytes at sites of inflammation is mediated by selectin molecules (Tedder et al., 1995). The adhesion molecules P-selectin, L-selectin and E-selectin may all contribute to monocyte adherence to endothelium (Kuijpers and Harlan, 1993).

L-selectin expressed on leukocytes and P-selectin and E-selectin expressed on vascular endothelial cells have been shown to mediate the tethering and rolling of leukocytes in shear flow (Albelda et al., 1994). The $\beta_1$-integrin vascular cell adhesion molecule (VCAM-1) has also been demonstrated to be important in monocyte adherence to vascular endothelium (Anderson and Springer, 1987). The integrin VLA-4, which is present on mononuclear leukocytes mediates tethering, independent of activation, to VCAM-1 (Alan et al., 1995). Transmigration of monocytes into the tissues requires the vascular expression of platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Albelda et al., 1994, Fawcett et al., 1995).

A potential monocyte-specific mechanism of adherence to endothelial cells has been suggested to occur via the CD14 antigen, a glycosphosphatidylinositol lipid-anchored glycoprotein on the surface of phagocytic cells. In relation to this, anti-CD14 antibodies inhibit the binding of monocytes but not neutrophils to cytokine-treated endothelial cells (Beekhuizen and van Furth, 1993). LPS binding studies have revealed that although $P. gingivalis$ LPS binds to CD14, human endothelial cells do not respond to $P. gingivalis$ LPS. It has been postulated that CD14-$P. gingivalis$ LPS complexes are not recognised by endothelial cells (Cunningham et al., 1999).

In the process of migration, monocyte chemoattractant proteins, MCP-1, MCP-2 and MCP-3, M-CSF, GM-CSF, TNF or transforming growth factor-beta (TGF-$\beta$) are capable of inducing monocyte/macrophage chemotaxis. During migration to the site of inflammation monocytes undergo differentiation into macrophages. The specific mechanism involved has still to be fully elucidated, however. There is evidence that the immediate extracellular environment of extravasating monocytes critically determines
the pathway of macrophage differentiation and activation. In this context, the macrophage-extracellular matrix interaction is important for the regulation of cytokine induction and release (Haskill et al., 1992).

3.3 FUNCTIONAL DIVERSITY

The extensive range of phenotypes displayed by macrophages may account for their vast functional plasticity (Rutherford et al., 1993). The traditional role ascribed to the macrophage is that of a scavenger cell involved in the removal of foreign agents, effete cells, and tissue debris. Macrophages also have decisive roles in acute and chronic inflammation (Riches, 1988 and Sorg, 1991), tissue turnover and repair (Leibovich and Ross, 1975, Riches, 1988), the immune response and tumour surveillance and killing (Russell et al., 1977, and Ruco and Meltzer, 1978).

Whether there are distinct specialised subsets within macrophage populations remains controversial. It has been established that only a limited number of macrophages perform a certain function at a given time. This does not confirm that a stable specialised subpopulation of macrophages occurs, rather this could be a reflection of fluctuations of function with time as a result of variations in stimulatory input.

Alternatively, distinct functional macrophage populations could provide the nonspecific immune system with greater flexibility to respond to challenge. Hence, it is probable that the nature of the immune response might largely be determined by the functional phenotypes of the macrophages present within the tissue or lesion. Alveolar macrophages, for example, have high cytotoxic and microbial potentials and are particularly effective as a nonspecific first line of defence against infectious agents, whereas lung interstitial macrophages have a greater capacity for a specific immune response, through secretion of cytokines such as interleukin-1 (IL-1) and IL-6, and accessory cell functions (Franke-Ullmann et al., 1996).

Importantly, macrophage function is determined to a large extent by signals received from the immediate environment. Whether these signals are directed at distinct macrophage populations, or at a pool of newly recruited macrophages arising from the constant influx of monocytes and/or by local proliferation and displaying a spectrum of maturational states, remains to be demonstrated.
3.4 MACROPHAGE FUNCTIONS IN CHRONIC INFLAMMATION

Macrophages perform important functions related to tissue defence in chronic inflammation. Also, in the process of chronic inflammation concurrent tissue destruction and ongoing repair occurs and the role of the macrophage assumes importance. In periodontitis, as in other chronic inflammatory diseases, the patho-physiological environment determines whether macrophages will perform cytotoxic, phagocytic, or secretory functions.

A selection of key macrophage functions relevant to chronic inflammation and potentially important in periodontitis is presented.

3.4.1 Scavenging activity

It is well established that macrophages have a scavenging role for the clearance of microorganisms, foreign particulate matter and altered-self materials such as apoptotic cells, senescent erythrocytes, immune complexes, and inflammatory products. This function is essential for tissue defence, maintenance and repair. Scavenging activity is based on phagocytosis and intracellular degradation or digestion. When microorganisms are ingested a microbicidal function is usually involved.

3.4.1.1 Phagocytosis

Phagocytosis is the process of recognition and engulfment of microorganisms or tissue debris that accumulate during infection, inflammation or wound repair. Phagocytosis, essential for successful host defense, is a highly selective process requiring specific interactions between the surface of the particle to be ingested and the plasma membrane of the macrophage. It requires a direct interaction between ligands on the particle and the phagocyte receptor, of which there are at least three types. The Fc IgG receptors that recognise the Fc portion of various subclasses of IgG molecules bound to a particle enables Fc-mediated phagocytosis in either activated or quiescent macrophages. Fc phagocytosis causes the release of toxic oxygen metabolites, arachidonic acid metabolites and other molecules. Complement phagocytosis receptors on activated macrophages recognise complement factors C3b, C3bi and C4b. Another
group of receptors directly recognises unusual oligosaccharide moieties not present in the host. These receptors include lectin receptors and lipopolysaccharide receptors.

Many molecules found at sites of inflammation or infection stimulate phagocytosis, so that efficient ingestion is confined to the site of infection or inflammation, which in turn limits the pro-inflammatory and tissue-destructive processes that accompany phagocytosis.

3.4.1.2 Microbicidal function

Following phagocytosis the microbe is sequestered inside the macrophage where it is exposed to multiple antimicrobial substances ranging in complexity from simple oxygen radicals to large proteins, nutrient deprivation, low pH, and digestive enzymes. This environment usually leads to disruption of various microbial structures resulting in death and degradation of the invading pathogen.

For those pathogens that are susceptible to attack by oxidizing agents, or free radicals, reactive oxygen intermediates and reactive nitrogen intermediates can be potent. These compounds release hydroxyl radicals and hydrogen peroxide. Longer-lived oxidants, such as hypochlorite, are formed after reactions between hydrogen peroxide and the pathogen. Another extremely powerful compound, nitric oxide, can also be released.

Another mechanism, the oxygen-independent killing mechanism, relies on a group of antipathogenic proteins known as defensins. These peptides form ion-permeable channels in bacterial cell membranes, leading to the shutdown of most cellular functions of the pathogen.

3.4.1.3 Intracellular degradation

A variety of hydrolytic and proteolytic enzymes are involved in degradation. Two hydrolase enzymes representing digestive capacity in activated macrophages are acid phosphatase (AP) and tartrate-resistant acid phosphatase (TRAP). These enzymes are described in a study investigating the digestive capacity of macrophages in periodontitis presented in Chapter 6.
3.4.2 Secretory function

Macrophages secrete over one hundred identified products ranging in biological activities from induction of cell growth to cell death. A single macrophage product can have multiple functions, while multiple products can effect a single activity. Other cell types can also secrete the same products as macrophages (Nathan, 1987). All this suggests the complexity of the regulatory mechanisms and interactions possible between the pleiotropic macrophage, other cells and the microenvironment. For example, when phagocytosis or certain soluble substances activate macrophages, they release cytokines, leukotriene B\textsubscript{4} and IL-8 that recruit neutrophils and lymphocytes to the site of inflammation. An excellent review of products secreted by the macrophage has been written by Nathan, 1987.

3.4.2.1 Growth factors and cytokines

Two growth factors secreted by macrophages, basic fibroblast growth factor and vascular endothelial growth factor, important in chronic inflammation and angiogenesis have been selected for discussion later in this chapter.

Macrophages are a major source of many cytokines involved in inflammation and immune responses, haematopoiesis, and many other homeostatic processes (Cavaillon, 1994).

Mackaness' concept of cell-mediated immunity laid the foundation for elucidating the mechanism of inborn resistance and acquired immunity. From this ensued the concept of various immune cell populations interacting via cytokines.

Chronic inflammatory cytokines can be subdivided into cytokines mediating humoral responses, for example, IL-4, IL-5, IL-6, IL-7, and IL-13, and those mediating cellular responses, such as IL-1, IL-2, IL-3, IL-4, IL-7, IL-9, IL-10, IL-12, interferons, TGF-\(\beta\), and TNF-\(\alpha\) and -\(\beta\). Some cytokines, for instance, IL-1, contribute significantly to both acute and chronic inflammation. Most cytokines have multifunctional roles, with some cytokines having similar effects, such as IL-1, IL-6 and TNF-\(\alpha\). Also, more than one cytokine can act on the same target cell.

The study of cytokines in chronic inflammatory diseases, such as periodontitis has been the focus of much research. This topic will be addressed in Chapter 4.
3.4.3 Antigen presentation

Activated macrophages display increased surface expression of major histocompatibility complex (MHC) class II molecules. MHC class II molecules are the key molecules that regulate cellular responses directed against protein or peptide antigens (Unanue and Allen, 1987). Class II molecules (also termed Ia molecules) are found mainly on the surface membranes of macrophages, B-cells, Langerhans cells and dendritic cells. These cells are referred to as antigen presenting cells (APCs) because of the antigen-processing and -presenting capabilities (Tykocinski et al., 1996).

Antigen presentation by macrophages involves a series of events. Particulate antigens are internalised and usually undergo intracellular processing, after which the antigenic fragment is displayed with the MHC class II molecules. CD4-positive T cells recognise the displayed proteins. The secretion of growth-differentiating molecules, such as IL-1 by the macrophage, is required to activate T cells. The activation of CD4-positive cells initiates the diverse cellular interactions that result in B cell activation, development of inflammatory reactions and activation of CD8-positive cells to become active killer cells. Dendritic cells are known as the most efficient antigen-presenting cell type to activate T cells. They are 100-fold more potent than macrophages as APCs (Di Nicola and Lemoli, 2000).

It is important to note that expression of MHC class II on macrophages is transitory, with positive expression eventually becoming negative, resulting in loss of antigen presenting capabilities (Unanue and Allen, 1987). The ratio of class II molecule-positive to -negative macrophages varies widely between different tissues. For example, peritoneal macrophages and spleen white pulp macrophages are mainly negative, whereas spleen red pulp macrophages are predominantly positive.

IFN-γ produced by T cells during antigen presentation, induces further MHC class II expression on macrophages. Under prolonged antigenic stimulation IFN-γ-induced expression of class II molecules is extended to other cells, such as fibroblasts, endothelial cells, and keratinocytes. These cells have been termed "non-professional" APCs.
3.5 ANGIogenesis

Chronic inflammation and angiogenesis appear to be co-dependent in the pathogenesis of chronic inflammatory diseases (Polverini, 1995, 1997, Jackson et al., 1997). Angiogenesis, the formation of new capillaries from pre-existing blood vessels, can maintain the chronic inflammatory state by directing inflammatory cells, nutrients and oxygen to the inflammatory site. Angiogenesis is essential for the formation of granulation tissue, tissue repair and remodelling (Sunderkotter et al., 1994).

Angiogenesis results from an orderly sequence of events. The formation of new capillaries starts with destruction of the basement membrane and local degradation of the extracellular matrix (ECM). This allows endothelial cells to migrate by extending cytoplasmic buds or sprouts in the direction of chemotactic factors. In inflamed tissue exudation of fibrin from leaking capillaries serves as a migratory matrix for endothelial and other cells. Elongation of new capillaries occurs by endothelial cell proliferation and maturation distant from the leading migratory cells. Studies in vitro indicate that newly forming vessels deposit the basement membrane components, laminin and type IV collagen independently (Yurchenco et al., 1992). Initial secretion of laminin is followed by type IV collagen at the same time as lumen formation occurs (Kramer et al., 1984, Nicosia and Madri, 1987). A comprehensive review of the physiology of angiogenesis is provided by Polverini, 1995.

3.5.1 Macrophages in angiogenesis

Macrophages have emerged as major protagonists for induction or modulation of angiogenic activity. Cell depletion studies have demonstrated that without macrophages new blood vessel formation is suppressed (Polverini and Leibovich, 1985, 1987). Other cells capable of angiogenic activity include keratinocytes (Detmar et al., 1994), granulocytes (Gaudry et al., 1997), mast cells in tumour angiogenesis (Blood and Zetter, 1990) and T lymphocytes in graft-versus-host reactions (Sidky and Auerbach, 1975). Activated lymphocytes release lymphokines that may act either directly by inducing endothelial cell migration or indirectly by stimulating macrophage activation (Auerbach and Sidky, 1979). The role of lymphocytes in vascular remodelling during chronic inflammation is unknown.
Importantly, macrophages must be activated in order to secrete angiogenic factors critical to angiogenesis and vascular remodelling (Polverini et al., 1977, Joseph-Silverstein and Rifkin, 1987). Alternatively, non-activated macrophages exhibit a non-angiogenic phenotype (Polverini et al., 1977, Polverini and Leibovich, 1985). Activation for angiogenesis is different from activation for antigen presentation. Koch et al., 1986 found however, that MHC molecules were expressed on angiogenic as well as non angiogenic macrophages in synovial tissue from rheumatoid arthritis patients. Other studies using macrophage-like cell lines have demonstrated that the capacity for angiogenic activity depends on the developmental stage of the macrophage (Polverini and Leibovich, 1985, Kodelja et al., 1997).

Macrophages can be activated to induce angiogenic activity by reduced oxygen tension (hypoxia) or wound-like concentrations of lactate, pyruvate, or hydrogen ions in the immediate environment (Xiong et al., 1998). Products such as IFN-γ, GM-CSF, MCP and LPS can also activate macrophages to secrete regulators of angiogenesis. Production of angiogenic activity by macrophages is dependent on a balance between positive angiogenic regulators and inhibitors. Positive angiogenic regulators produced by activated macrophages include TNF-α, TGF-α and -β (Roberts et al., 1986, Joseph-Silverstein and Rifkin, 1987), and IL-8; angiogenic inhibitors include thrombospondin, angiostatin, TGF-β (Sunderkotter et al., 1991) and IFN-α. Currently, there is intensive investigation to elucidate the mechanisms controlling the regulators of angiogenic activity.

Macrophages are able to promote all phases of the angiogenic process by virtue of secretory products (reviewed in Sunderkotter et al., 1994). Macrophages are a rich source of metalloproteinases (e.g. collagenases) and serine proteinases (e.g. elastase and plasminogen activator) that can degrade ECM molecules and liberate ECM-bound growth factors such as, basic fibroblast growth factor (bFGF) and GM-CSF.

As proteolytic enzymes are capable of degrading almost all components of the ECM, control mechanisms are necessary. Macrophages synthesise tissue inhibitors of metallo- and serine proteinases. TGF-β, which both stimulates and inhibits angiogenesis in vitro (Joseph-Silverstein and Rifkin, 1987, Sunderkotter et al., 1991), modulates the
expression of fibronectin or collagen type I and the incorporation into the ECM. Other
macrophage-derived cytokines with angiogenic potential and modulating effects on the
ECM are angiotropin, platelet-derived growth factor (PDGF), and IL-6 (Polverini,
1996).

A model of wound angiogenesis has been proposed by Nissen and coworkers, in
which an initial angiogenic stimulus supplied by bFGF is followed by a subsequent and
more prolonged angiogenic stimulus mediated by VEGF (Nissen et al., 1998). It has
been suggested that a constant presence of VEGF may be required to maintain the
differentiated state of microvessels and that suppression of VEGF may contribute to
vessel regression (Ferrara and Davis-Smyth, 1997) and tissue disruption. These two
potent macrophage angiogenic growth factors will be discussed in more detail.

3.5.1.1 Basic fibroblast growth factor (bFGF or FGF-2)

Basic FGF belongs to a family of heparin-binding, single-chain polypeptide growth
factors and is a potent mitogenic and chemotactic factor for endothelial cells and
fibroblasts. It stimulates directed migration and proliferation of cultured endothelial cells
and promotes formation of differentiated capillary tubes in vitro. These effects are
associated with selective up-regulation of integrins on endothelial cells and induction of
proteinases such as plasminogen activator.

Immunolocalisation of bFGF in situ has shown a conspicuous expression in
inflammatory tissues (Schulze-Osthoff et al., 1990) as well as in a variety of
physiologically normal tissues (Cordon-Cardo et al., 1990). Basic FGF is expressed by
macrophages (Schulze-Osthoff et al., 1990), basal keratinocytes, sweat and sebaceous
glands, dendritic cells of the thymus (Schulze-Osthoff et al., 1990, Hughes and Hall,
1993), and tissue vasculature (Cordon-Cardo et al., 1990, Hughes and Hall, 1993). Basic
FGF lacks a signal sequence and yet it is found localised in the ECM where it is bound
to heparin sulphate. Heparin has been shown to protect bFGF from proteolytic
degradation. Heparinases and plasmin, both secreted or activated by macrophages are
able to release bFGF from the ECM, thus enabling bFGF to reach its receptors on
endothelial cells (Sunderkotter et al., 1994).
3.5.1.2 Vascular endothelial growth factor (VEGF)

VEGF has emerged as a central regulator of the angiogenic process in physiological conditions, such as wound healing, and under pathological circumstances, such as chronic inflammation (reviewed by Dvorak et al., 1995, Ferrara and Davis-Smyth, 1997). VEGF is expressed by macrophages (Berse et al., 1992), keratinocytes (Brown et al., 1992), neutrophils (Taichman et al., 1997) serous acinar cells and ductal epithelial cells (Taichman et al., 1998).

VEGF represents a family of secreted growth factors that act specifically on vascular endothelial cells. VEGF is a heparin-binding glycoprotein that occurs in four main isoforms, consisting of 121, 165, 189, and 206 amino acids derived from the same gene by alternative splicing of the gene transcript. VEGF binds to two high-affinity tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1, or KDR), which are expressed primarily on endothelial cells.

VEGF stimulates proliferation of endothelial cells; it enhances vascular permeability to fluids and plasma proteins (reviewed by Dvorak et al., 1995); it stimulates endothelial cells to release von Willebrand factor and interstitial collagenase; it up-regulates plasminogen activator, plasminogen activator inhibitor-1, and nitric oxide; and induces serine- and metallo-proteinase expression by endothelial cells. These VEGF-mediated events have been implicated in the progression of inflammation and wound healing (reviewed by Brown et al., 1997).

3.6 THE STUDY OF MACROPHAGES

Differences in surface phenotype, biochemistry, secretory products, functions, and morphology determine the overall phenotypic appearance of macrophages, which differs depending on the location in different tissues. In this context, the behaviour of extracted macrophages in vitro may reflect a functional phenotype specific to the tissue of origin and may not resemble that of other macrophages. When macrophages are released from environmental constraints for study in vitro, their behaviour may bear little resemblance to the behaviour in situ (Sorg, 1991). Furthermore, the functional state of the macrophage isolated for study in vitro may be a result of the stimulatory effect of the extraction procedure employed (Malorny et al., 1981).
Murine peritoneal macrophages have often been used in studies in vitro as representative of human inflammatory macrophages. This assumption cannot be validated and an interpretation relating to human macrophage behaviour in vivo is not appropriate.

3.6.1 Identification in vivo

Early studies used morphological features to identify macrophages in situ. A typical macrophage is large, up to 50μm in diameter, with an ovoid or elongated shape, however it displays a diverse appearance that is dependent on the stage of maturity and activity. Accurate identification using light microscopy is difficult unless the cell displays obvious evidence of phagocytic activity, such as the inclusion of visible ingested material within the phagolysosomes. A distinctive feature is the indented or kidney-shaped nucleus; however, the plane of sectioning is critical for its detection. On the basis of morphological criteria alone macrophages can be confused with other cells in the connective tissue such as reticulum cells, dendritic cells and fibroblasts (van Furth, 1981).

At the transmission electron microscope level the surface of the mature and activated macrophage displays numerous folds and finger-like projections. Other features include large golgi, rough and smooth endoplasmic reticulum, mitochondria, secretory vesicles and lysosomes. Although electron microscopy enables macrophage identification at an ultrastructural level, this technology is usually employed as an adjunct to light microscopic study whereby macrophages are investigated in relation to tissue structures.

Other markers used to identify macrophages are associated with the function of phagocytosis and degradation, such as acid-phosphatase and non-specific esterase, which can be visualised by enzyme histochemistry (Danenberg and Suga, 1981). These enzymes although produced in abundance by phagocytosing macrophages, are not macrophage specific.

With the development of the technique to produce monoclonal antibodies (Kohler and Milstein, 1975) more powerful tools to study the diverse heterogeneity and functional states of macrophages both in vivo and in vitro have become available. There are more than 40 monoclonal antibodies directed against human mononuclear phagocyte
antigens. Comprehensive reviews are available for many of these markers (Ho and Springer, 1984, Hogg, 1987, Gordon, 1999). Determining the functional significance of the many surface antigens on human macrophages is the focus of intensive current research.

At present, it is not known whether many of the monoclonal antibodies used to identify human macrophages are distinguishing surface molecules associated with stage of differentiation, maturation, or function. Specificity is also a concern as many markers identify a range of cell types within the mononuclear phagocyte system. As monoclonal antibodies recognise a single determinant on a macromolecule, the detection of an antigen on the cell surface may depend on factors such as synthesis, turnover, masking (Hirsch and Gordon, 1982) and preservation. A number of different genes are usually involved in the production of an antigen and factors such as proteolytic enzymes and interactions with the extracellular milieu could profoundly influence the expression of the antigen (Hirsch and Gordon, 1982).

3.6.2 Monoclonal antibodies

Monoclonal antibodies have been used extensively to define macrophages in normal and diseased tissues and to identify the states of differentiation, maturation, activation (Hirsch and Gordon, 1982) and function. Most of the antigens recognised by these monoclonal antibodies are glycoproteins present on the cell surface membrane or on membranes of cytoplasmic organelles.

CD68 and CD14 are useful macrophage markers but are less specific than CD163, 27E10 and 25F9. In the past, CD68, a pan macrophage marker, was the most commonly used monoclonal antibody, followed by CD14, for identifying macrophages in periodontal tissues.

The monoclonal antibodies Ber-MAC3 (anti-CD163), RM3/1 (anti-CD163), 27E10 and 25F9 are used in the studies presented in later chapters. These phenotypic macrophage markers were selected on the basis of their high specificity. A summary of the specificities of monoclonal antibodies used in the studies is provided in Table 1.
Preliminary studies indicated that the anti-CD14 antibody tested has poor specificity. Therefore, CD14 was not used in the study but has been included in the following description of monoclonal antibody markers.

3.6.2.1 CD163 (Ber-MAC3 and RM3/1)

The monoclonal antibodies, Ber-MAC3, Ki-M8, GHI/61 and SM4 define a human macrophage-associated antigen with a relative molecular mass of 130,000, designated M130 (Law et al., 1993). Human transmembrane glycoprotein M130 was assigned to CD163 at the 6th Human Leukocyte Typing Workshop. Recently, RM3/1 has also been assigned to CD163. Ki-M8 and RM3/1 recognise different epitopes of the CD163 antigen.

3.6.2.1.1 Structure of CD163

CD163 is a member of the scavenger receptor/cysteine-rich (SRCR) superfamily, group B proteins. It is restricted to cells of the mononuclear phagocyte lineage where it is found on all circulating monocytes and most tissue macrophages (Radzun et al., 1987). CD163 is located on the cell surface and in an intracellular location (Pulford et al., 1989).

The genomic organization and chromosomal localisation of the human CD163 gene have recently been determined (Law et al., 1993; Ritter et al., 1999). The CD163 gene has been mapped to region p13 on chromosome 12. To date, no other member of the SRCR-subfamily has been found at this chromosomal site. There are several putative binding sites for transcription factors which have been shown to play an important role in myeloid-specific gene expression and differentiation. Also identified are three putative glucocorticoid receptor-binding sites. These may contribute to the strong induction of CD163 mRNA and protein expression following glucocorticoid treatment of monocytes and macrophages in vitro (Zwadlo et al., 1987, Hogger et al., 1998).

3.6.2.1.2 Isoforms of CD163

The CD163 extracellular domain is composed of nine SRCR-domains followed by a transmembrane segment of 24 amino acids and a short cytoplasmic tail (Law et al., 1993). Two cytoplasmic variants of CD163 arise from alternative splicing of intron 15, while a truncated and an extracellular variant results from alternative splicing of intron 5
or intron 7, respectively. Variant 2 is found at significant levels in monocytes and macrophages (Law et al., 1993, Ritter et al., 1999). This splice variant involves some kind of splice site repression. The truncated variant might be secreted from the cell (Law et al., 1993, Ritter et al., 1999), however, the existence of this protein has still to be demonstrated. There appears to be no correlation between variation in the molecular size of the CD163 antigen and the expression of a particular variant mRNA. It has not been determined whether Ber-MAC3 and RM3/1 are different cytoplasmic variants.

3.6.2.1.3 Functions of CD163

CD163 is presumed to be a receptor, however the precise function is unknown. Several observations reveal an important role of this protein in the regulation of the immune response by monocytes and macrophages. The expression of CD163 is tightly controlled by pro- and anti-inflammatory stimuli. Pro-inflammatory stimuli, such as lipopolysaccharides and interferon-γ, suppress the CD163 mRNA and protein expression whereas anti-inflammatory mediators, including the glucocorticoid dexamethasone and interleukin-10, induce rapid upregulation of CD163 mRNA and protein (Zwadlo et al., 1987, Kodelja et al., 1994). In addition, CD163 mRNA is upregulated during phagocytic differentiation of mononuclear cells, in contrast to dendritic differentiation where the expression of CD163 is suppressed (Ritter et al., 1999). A marked increase in MAC3 expression occurs at the differentiation step related to monocyte emigration in vivo, and in vitro (Law et al., 1993).

Significantly, “scavenging” activities are unlikely to be mediated by the so-called SR-domain and thus the name “scavenger” may be misleading. Evidence for this is based on a more defined consensus of the primary structure of the scavenger receptor domains of CD163 (Law et al., 1993) and WC1 antigens (Matsudaira, 1987). WC1 antigen is expressed exclusively on the cell surface of CD4-, CD8-, gamma delta T lymphocytes. The two forms of scavenger receptors differ by the presence or absence of a single SR-domain, and are functionally indistinguishable.

Previous functional studies have suggested that the antigen could be associated with the generation of oxygen radicals, however, this observation has not been supported by subsequent work. It has also been speculated that CD163 is a receptor for monocyte
chemoattractant protein 1 (MCP-1). There is no evidence to support this proposed function.

Although the precise functions of the different isoforms have yet to be clarified, the altered cytoplasmic domains of variant 1 and 2 suggest that these isoforms could modulate cell signalling through CD163. At present, no functional differences have been associated with these isoforms (Ritter et al., 1999, Law et al., 1993).

Characterisation of the signalling capabilities of the cytoplasmic isoforms of CD163 and identification of the corresponding ligands is considered to be crucial for elucidating the functions of the CD163 isoforms in the inflammatory response.

3.6.2.2 27E10

27E10 has been described as an acute inflammatory marker. Antibody 27E10 detects a 17kDa surface and cytoplasmic antigen found on macrophages in inflammatory foci (Zwadlo et al., 1986), on monocytes, neutrophilic granulocytes localised in blood vessels (Betz et al., 1995), and keratinocytes (Iacopino et al., 1997). It is not found in HLA-DR-positive interdigitating cells (Brandtzaeg et al., 1987). The 27E10 antigen is the heterodimer MRP8/MRP14 complex formed by non-covalent association of the two Ca(2+)-binding proteins MRP8 and MRP14 which belong to the S100 protein family (Bhardwaj et al., 1992).

3.6.2.2.1 Functions of 27E10

Antigen expression can be increased by exposure to lipopolysaccharide, interferon-γ and phorbol esters, suggesting that the antigen recognised by 27E10 is expressed as a component of activation. Furthermore, 27E10 surface-positive monocytes in vitro release high amounts of TNF-α and IL-1β, thus supporting the role of 27E10 positive macrophages in inflammation (Bhardwaj et al., 1992).

Although the function of 27E10+ macrophages in inflammation remains to be determined, more is known about the MRP8/MRP14 complex. The MRP8/MRP14 complex is also commonly known as calprotectin, while other terms include leukocyte-derived protein (L1) light and heavy chains, p8/p14, calgranulin A/B, and the cystic fibrosis antigen. The soluble form of calprotectin is released from macrophages and
neutrophilic granulocytes during activation or cell death. Calprotectin is found in body fluids in inflammatory conditions and thus may be considered as a sensitive inflammation marker. This has been demonstrated recently in a study of the level of calprotectin in gingival crevicular fluid collected from healthy and periodontitis sites (Kido et al., 1998). Data revealed high correlation between increased levels of calprotectin and increased probing depths and positive bleeding on probing, and with elevated levels of IL-1 and PGE2 (Kido et al., 1999). It has also been reported that keratinocyte expression of MRP8/14 is elevated in oral inflammatory mucosal diseases of immunopathological, fungal and viral origin, such as lichen planus, candidiasis, herpes virus stomatitis, and oral hairy leukoplakia (Eversole et al., 1993). In addition to evidence of antibacterial, antifungal and anti-proliferative activities in vitro (Murao et al., 1990, Miyasaka et al., 1993, Murthy et al., 1993) another important biological function recently identified for this protein is the capability for fatty acid transport (Siegenthaler et al., 1997).

3.6.2.3 25F9

25F9, described as a resident macrophage marker, is an 86kDa antigen found on macrophages and keratinocytes in normal and diseased skin (Simon et al., 1993). The antibody is unreactive for monocytes, granulocytes, lymphocytes and epidermal Langerhans cells (Zwadlo et al., 1985). Interferon-γ, and to a lesser degree TNF-α, inhibit expression of 25F9 antigen in cultured human monocytes and macrophages (Kodelja et al., 1994). Antibody 25F9 reacts strongly with mature, non-inflammatory macrophages in a variety of tissues (Zwadlo et al., 1985) and is also expressed on basal keratinocytes in gingival tissue (Schlegel-Gomez et al., 1995) but is absent from blood monocytes (Zwadlo et al., 1985).

3.6.2.3.1 Functions of 25F9

25F9 is regarded as a very late macrophage differentiation antigen (Zwadlo et al., 1986). Hume et al., (1987) have however, interpreted the results of studies in vivo, suggesting that 25F9 may be a marker not of maturation but of endocytic history.
3.6.2.4  Anti-CD68

CD68 is a useful pan macrophage marker but with lower specificity than the previously described antigens. CD68 is a 110 to 120-kDa transmembrane glycoprotein and is a member of the lysosomal-associated membrane protein [lamp] family with a mucin-like extracellular domain (Martinez-Pomares et al., 1996). CD68 specificity for macrophages is not as high as CD163. CD68 is expressed specifically in a wide variety of tissue macrophages, including Kupffer cells, splenic, lamina propria macrophages, and myeloid derived cells, such as Langerhans cells and interdigitating reticulum cells (Holness and Simmons, 1993) as well as neutrophils and granulocyte precursors (Pulford et al., 1989).

The CD68 antigen is found in monocytes within cytoplasmic granules co-localised with lysozyme and myeloperoxidase. In neutrophils the antigen is found in the primary granules but is absent from the plasma membrane (Saito et al., 1991).

In the study described in Chapter 5 the monoclonal antibody KP1 is used to detect CD68 antigen. KP1 is raised against a lysosomal fraction of human lung macrophages and recognises a fixation-resistant epitope in a wide variety of tissue macrophages. KP1 and antibodies EBM11, Y1/82A, Y2/131 and Ki-M6 appear to recognise different epitopes on the CD68 antigen (Holness and Simmons, 1993).

3.6.2.4.1  Functions of CD68

The function of CD68 has not been fully elucidated. While there is mounting evidence to support a role for CD68 in lipoprotein regulation as a specific oxidised low-density lipoprotein (LDL) binding protein in human monocyte-derived macrophages (Van der Kooij et al., 1997, Jiang et al., 1998), it may play a role in endocytosis or lysosomal trafficking (Holness and Simmons, 1993).

3.6.2.5  CD14

CD14 antigen is a plasma membrane LPS receptor for the binding of a complex of LPS with LPB (LPS-binding protein). CD14 antigen is one of at least three receptors that recognise LPS. Other known plasma membrane receptors for LPS are CD18 and the scavenger receptor, also called acetyl LDL-receptor.
There are at least three different forms of CD14, which may reflect different stages of cell maturation (Pedron et al., 1995).

Some CD14 monoclonal antibodies react with monocytes, dendritic reticulum cells and some endothelial cells while others are more broadly reactive, labelling in addition to the aforementioned, tonsil Langerhans cells, interdigitating cells, some granulocytes and even basophils. More is known about the 55kDa LPB protein with which CD14 reacts.

Table 1 Specificities of monoclonal antibodies used in studies and for CD14.

<table>
<thead>
<tr>
<th>Specificity (CD163)</th>
<th>Description</th>
<th>Function/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ber-MAC3</td>
<td>activated monocytes, macrophages</td>
<td>specific differentiation stage macrophages</td>
</tr>
<tr>
<td>RM3/1</td>
<td>10-20% monocytes, macrophages</td>
<td>anti-inflammatory, reparative macrophages</td>
</tr>
<tr>
<td>27E10</td>
<td>10-20% monocytes, macrophages, neutrophilic granulocytes, keratinocytes</td>
<td>acute inflammatory macrophages</td>
</tr>
<tr>
<td>25F9</td>
<td>macrophages, keratinocytes</td>
<td>very late stage differentiation macrophages, resident macrophages</td>
</tr>
<tr>
<td>CD68</td>
<td>monocytes, macrophages, myeloid derived cells eg Langerhans cells, interdigitating reticulum cells, neutrophils, granulocyte precursors,</td>
<td>pan-macrophages</td>
</tr>
<tr>
<td>CD14</td>
<td>monocytes, dendritic reticulum cells, interdigitating cells, some endothelial cells, some granulocytes, basophils</td>
<td>myeloid differentiation antigen</td>
</tr>
</tbody>
</table>
3.6.3 Confocal microscopy

Limitations are imposed on the interpretation of histopathological and immunohistochemical observations. For example, in morphometric analysis of frozen tissue sections a potential error is introduced as more than one plane can be viewed at one time. This problem is compounded as sectioning produces subtle variable thicknesses within each cut section. Optical sectioning using confocal microscopy overcomes this problem (Ockelford, 1995, Paddock, 1996).

Confocal technology employs a laser to deliver concentrated light to a specimen at controllable wavelengths. The resulting reflection (fluorescence) is passed back along the optical path and through a small aperture, the confocal pinhole, before reaching an electronic detector or photomultiplier tube (PMT). The PMT converts the light into a digital image that is then displayed on a computer monitor. The confocal pinhole permits only light returning from an exact plane of focus to pass through to the PMT. Light returning from planes other than the specific optical plane is blocked by the pinhole and omitted (Figure 1A). This process creates an extremely thin optical section ($x, t$ scan) that can be positioned anywhere within a specimen to provide an image that has a significantly higher resolution than a conventional microscope.

The confocal system can combine multiple optical sections into an "optical stack" ($z$ series) to produce a three-dimensional (3-D) image, making this technology appropriate for the study of detailed 3-D structural aspects of macrophages and the distribution and spatial relation of these cells to the surrounding environment (Paddock, 1996).

Confocal laser scanning microscopy (CLSM) permits accurate co-localisation of fluorescent markers, because the thin optical section generated by the instrument eliminates the confounding effects of out-of-focus fluorescence (Wouterlood et al., 1998). As in conventional dual immunofluorescence, detection of co-localisation requires appropriate controls to ensure the absence of cross-reactivity and of non-specific binding of antibodies or other fluorochrome-labelled markers. In addition, technical issues unique to CLSM must be specifically addressed.

For valid interpretation of the digital images generated by CLSM, reliable separation of the signals generated by the fluorochromes is critical. This is in turn dependent on the respective excitation/emission spectra and the choice of barrier filters. Overlap between
Figure 1A Principal design (optical path) of a confocal microscope. Light emitted from outside the focal planes does not reach the detector and is not recorded.

Figure 1B Optiscan F900e personal confocal system used in the studies presented in this thesis. Diagram provided by OptiScan Pty Ltd, Melbourne, Australia.
the emission spectra of fluorescein and tetramethylrhodamine renders simultaneous excitation of probes labelled with these dyes unsatisfactory for CLSM (Entwistle and Noble, 1992).

In a collaborative study, this writer, RK Kumar and N Hunter (Kumar et al., 1999) have successfully overcome the limitation of a single argon ion laser (Optiscan F900e, Figure 1B) in achieving effective excitation of dyes with well-separated emission spectra by employing the novel sulphonated rhodamine fluorochromes designated Alexa 488 and Alexa 568. The more abundant antigen was visualised using the red-emitting Alexa 568, with amplification of the signal by a biotinylated bridging antibody and labelled streptavidin. This was combined with the green-emitting Alexa 488, which yielded brighter images than fluorescein but exhibited comparable photodegradation. With appropriate controls to ensure the absence of cross-talk between fluorescence channels, these dyes permitted unequivocal demonstration of co-localisation.

The improved dual immunofluorescence technique for single argon ion CLSM presented in the paper is used, and where appropriate, in combination with 3-D reconstruction, in the studies described in Chapters 7 and 8.

3.7 STUDY OF MACROPHAGES IN PERIODONTITIS IN VIVO

Dual immunofluorescence with high specificity monoclonal antibodies and confocal microscopy are valuable tools for the investigation of macrophages in vivo and are used in the studies described in Chapters 7 and 8. The studies presented in Chapters 5 and 6 employ various combinations of established single and dual immunohistochemistry and histochemistry techniques. Morphometry is employed for quantification and minimally inflamed gingival tissue used as a control and for comparison. For good antigen preservation carefully prepared frozen tissue sections of untreated advanced periodontitis, representative of the natural destructive disease, are used.

In the following chapter the chronic inflammatory disease periodontitis is reviewed.
CHAPTER 4
PERIODONTITIS

4.1 INTRODUCTION

Periodontal diseases are widespread in the population with up to 15% of adults developing progressive, destructive lesions, which constitute a major cause of tooth loss and dysfunction of the dentition (Position paper: Epidemiology of periodontal diseases. American Academy of Periodontology, 1996, Papapanou, 1996).

4.1.1 Classification of periodontal diseases

The term periodontal disease defines a group of chronic inflammatory diseases that are localised to the tissues of the periodontium.

At the World Workshop on Clinical Periodontics in 1989, a reclassification of periodontal diseases was suggested. The following disease entities were primarily based on clinical characteristics and include: Adult Periodontitis; Early-Onset Periodontitis comprising Prepubertal Periodontitis, Juvenile Periodontitis (Generalised and Localised) and Rapidly Progressive Periodontitis; Periodontitis Associated with Systemic Disease; Necrotizing Ulcerative Periodontitis; and Refractory Periodontitis. The defining clinical characteristics were age of onset, distribution of defects within the dentition, rate of progression, relation to systemic disease, and response to treatment.

More recently, the European Academy of Periodontology (Proceedings of the first European workshop on Periodontology, 1994) and the American Academy of Periodontology (Armitage, 1996)*, acknowledged that there existed an extensive overlap between various disease forms in the classification system. Furthermore, several disease subentities display similar microbiological- and host- response features. Importantly, it was recognised that there was insufficient knowledge to distinguish different diseases from differences in presentation of the same disease. The accumulating data on

* Postscript: After preparation of this thesis the proceedings of the "1999 International Workshop for a Classification of Periodontal Diseases and Conditions" was published (Annals of Periodontology 4: December 1999).
microbiological and pathogenetic characteristics of the different disease entities have the potential to redefine the current classification.

The studies in this thesis are concerned with the most prevalent form of periodontitis, adult periodontitis, characterised by an onset from about 35 years and a slow net rate of progression. Only biopsies from donors with untreated advanced periodontitis, over the age of 35 years were used. The collected tissues were considered to be representative of the native destructive lesion. The approach taken was to compare minimally inflamed tissues with advanced destructive disease, that is the extremes of presentation.

4.2 ADULT PERIODONTITIS

Adult periodontitis is an immunopathological response induced by a complex oral microflora. Pathological features of this disease include, progressive destruction of the connective tissue matrix, characteristic and manifest vascular pathology and the accumulation of immune system cells, accompanied by failure of the epithelial attachment to the tooth. This results in the formation of a pocket lined by epithelium in close proximity to a complex microbial biofilm.

The exact mechanism for pocket development is unclear, although bacteria or their products appear to be necessary for the formation of pocket epithelium (PE). Breakdown of the epithelial attachment and apical re-positioning along the tooth root is assumed to occur by migration of the cells of the attachment analogous to the behaviour of epithelium in wound healing (Gao and Mackenzie, 1992). Animal studies have demonstrated that when bacterial products are injected subdermally or are applied directly to gingival tissue, epithelial cell proliferation and migration occurs (Hunter et al., 1979, Suzumura, et al., 1989, Takata et al., 1997). Further, synthesis of metalloproteinase XIII by the lining epithelium has been described (Uitto et al., 1998) with implication for the destructive potential of this tissue. Altered barrier function of the pathological lining epithelium could critically influence the ingress of microbial products into the tissues and therefore regulate the pathogenesis of destructive periodontitis.
The low redox potential of the deepening pocket creates an environment favourable for the abundant growth of microorganisms in a highly organised biofilm. The subgingival biofilm comprises a tightly adherent structure to the root surface as well as loosely adherent and unattached bacteria in close proximity to the pocket epithelium. Although the behaviour of biofilms is not fully understood, it is recognised that these complex microbial structures are able to resist the usual host defences (Darveau et al., 1997). Also, virulence characteristics of species in close association with other species can alter, for example, *Bacteroides forsythus* is highly virulent and invasive in combination with *Porphyromonas gingivalis* (Takemoto et al., 1997).

Over 300 species have been isolated from periodontal pockets. Less than 5% of species comprising the oral flora have been consistently associated with periodontitis and only a few species possess the virulence characteristics capable of disrupting the protective mechanisms of the host. There are two Gram-negative anaerobic pathogens, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (Zambon, 1996), that have a strong association with adult periodontitis.

Recent studies have identified *P. gingivalis* within pocket epithelium and in the deeper layers of the pocket wall in vivo (Noiri et al., 1997). Studies in vitro have demonstrated the ability of *P. gingivalis* to disrupt epithelial cell functions, and to invade and replicate within gingival epithelial cells (Lamont et al., 1995) and in endothelial cells (Deshpande et al., 1998). Cysteine proteinases from *P. gingivalis* can inhibit expression of MHC class II proteins on stimulated endothelial cells by cleavage of IFN-γ, resulting in potential disruption of recruitment and activation of leukocytes in the inflammatory response (Yun et al., 1999). *A. actinomycetemcomitans* can adhere to and invade epithelial cells in vitro and has the capacity to penetrate the deeper connective tissue (Meyer and Fives-Taylor, 1997). *A. actinomycetemcomitans* produces a leukotoxin, a member of the RTX toxin family of pore-forming hemolysins/leukotoxins (Lally et al., 1989) that kills human polymorphonuclear cells (PMNs) and monocytes (Taichman et al., 1980, Rabie et al., 1988). The effects of this suppression may enhance the pathogenicity of *A. actinomycetemcomitans* or that of some other opportunistic organism. The capacity for these two bacteria to modulate host immune processes has
been extensively studied \textit{in vitro} but whether these mechanisms are operational \textit{in vivo} has yet to be demonstrated. Comprehensive reviews of the potential pathogenic mechanisms of these putative periodontopathogens have been published by Meyer and Five-Taylor, 1997 and Lamont and Jenkinson, 1998.

\textbf{4.3 PATHOGENESIS OF PERIODONTITIS}

The pathogenesis or development of periodontitis is generally regarded as a sequence of events from health to severe periodontal tissue destruction. The same basic pathological mechanisms underlie all forms of bacterially-induced periodontal diseases (Page \textit{et al.}, 1997).

\textbf{4.3.1 Minimally inflamed gingiva}

In health, the gingiva in the adult human is characterised by a firm consistency, scalloped gingival margin, lack of bleeding when gently probed and a shallow sulcus depth usually 3mm or less (Figure 2). The colour of the gingiva may reflect the level of skin pigmentation and therefore varies. Normally, very little or no plaque is detectable clinically at the gingival margin.

Histologically, a small but definite infiltrate of inflammatory cells, namely, neutrophils (PMNs), lymphocytes and macrophages can be detected subjacent to the junctional epithelium (Figure 3). These cells reflect a normal host defence against a low-grade microbial challenge and tissue destruction does not occur. In this context clinically healthy gingiva is more appropriately referred to as minimally inflamed gingiva (Page and Schroeder, 1976).

\textbf{4.3.2 Gingivitis}

Within a few days of undisturbed bacterial growth on the cervical portion of the tooth surface, inflammatory changes develop in the adjacent gingiva. Clinically, these changes may be seen as discrete alterations in colour and consistency. If plaque accumulation continues, by 10 to 20 days, gingivitis is established in most individuals (Löe \textit{et al.}, 1965).

The classical clinical presentation is a deepening of gingival colour and swelling (Figure 2), resulting in alterations of gingival contour and consistency, and bleeding upon gentle probing. Although variations of presentation of inflammation may occur in individuals, bleeding upon gentle probing has been demonstrated in clinical and histological studies to be a sensitive indicator of gingival inflammation (Meitner \textit{et al.}, 1979).
Figure 2  Gingival tissue in area A displaying features of clinical health with no bleeding on probing, no alteration in colour and shallow sulcus depth. Gingival tissue in area B displaying features representative of gingivitis with alteration in gingival colour and margin contour associated with inflammation.
Figure 3  Minimally inflamed gingival biopsy section stained with haematoxylin and eosin showing oral epithelium (OE), connective tissue (CT) and oral sulcular epithelium (OSE) with a small inflammatory cell infiltrate (arrow).
4.4 A MODEL OF PATHOGENESIS

The progressive histological changes observed in the development of experimentally induced periodontitis in beagle dogs have formed the basis for a widely accepted model describing the development of gingivitis and periodontitis in humans (Page and Schroeder, 1976). The pathogenesis has been separated into the initial, early, established lesions that are overlapping, sequential stages in gingivitis and the advanced lesion that is manifested clinically as periodontitis. The initial and early lesions are acute inflammatory phases comparable to that elicited in most other tissues subjected to acute injury. These lesions are a consequence of the elaboration and release of chemotactic and antigenic substances by microbial plaque (Page and Schroeder, 1976). The established and advanced lesions are predominantly chronic phases with superimposed acute elements of inflammation. This is generally considered to be in part due to the dynamics of the evolving microbial biofilm and host responses to these changes.

4.4.1 Initial lesion

In the initial lesion, gingival tissues respond within 2 to 4 days to the accumulation of microbial plaque (Løe, et al., 1965), with a classic acute exudative vasculitis occurring in the microvascular plexus subjacent to the junctional epithelium. Exudation of fluid from the gingival sulcus and an increased migration of neutrophils (PMNs), lymphocytes and macrophages from vessels into the tissues occur. Small quantities of collagen and other components of the perivascular extracellular matrix are lost. The initial lesion can be induced experimentally by application of extracts of plaque bacteria to normal gingiva thus implicating bacterial constituents in effecting these changes.

4.4.2 Early lesion

The early lesion is characteristic of lesions seen at sites of cell-mediated hypersensitivity reactions. In the child and adolescent it develops usually within 4 to 7 days of plaque accumulation and is characterised by a lymphoid cell infiltrate dominated by T lymphocytes, transmigrating PMNs, as well as infiltrating macrophages and plasma cells (Schroeder and Listgarten, 1997). Pathological alteration of fibroblasts and
continuing loss of the connective tissue matrix occurs. The basal cells of the junctional epithelium start to proliferate. In adults this lesion is not distinctly identifiable histologically (Fransson et al., 1996, Page et al., 1997).

The early lesion can be induced by the application of purified contact antigens to the gingival tissues of previously sensitized animals (Page, 1986). In most individuals the early lesion is manifested clinically as gingivitis.

4.4.3 Established lesion

The established lesion appears at a variable time (Brecx et al., 1987), predominated by B lymphocytes and plasma cells which are not restricted to the region adjacent to the junctional epithelium but are found in clusters associated with blood vessels and deep within the connective tissues. Some plasma cells show signs of degeneration (Freeman et al., 1968). The inflammatory infiltrate also consists of macrophages and emigrating PMNs as well as T cells of both the T-helper (Th1) and Th2 phenotype. Connective tissue loss continues and proliferation of the junctional epithelium, with apical and lateral extension, occurs resulting in the formation of pocket epithelium (Figure 4). At this stage there is no loss of attachment or loss of bone. The established lesion may remain stable for indefinite periods of time.

Most observations of the pathogenesis of gingivitis have been based on either experimentally induced periodontitis in animal or short-term human experimental gingivitis studies. Brecx et al., 1988, however, examined long-term experimental gingivitis in young adult males. At six months a slow shift in the proportions of some cell populations was consistently observed. While the proportions of PMNs, lymphocytes and macrophages remained stable, a significant decrease of fibroblasts and an increase of plasma cells (0.2 to 10%) was observed. Whether these changes represent a contained or progressive lesion is not known.

The effect of age on the development of gingivitis in humans and dogs has been studied. In older humans, gingivitis was found to be more pronounced and the lesion contained more inflammatory cells (Fransson et al., 1996). In older dogs, the cellular
Figure 4 Gingivitis tissue biopsy section stained with haematoxylin and eosin showing inflammatory cell infiltrate (ICI) adjacent to OSE and proliferated pocket epithelium (PE).
infiltrate was also larger, extended more apically, and contained significantly more plasma cells but fewer macrophages, lymphocytes and PMNs than the infiltrate in young dogs (Berglundh and Lindhe, 1993). Whether these differences are a reflection of an ageing immune response or due to more occasions of plaque exposure over time can only be speculated. However, of particular relevance to the work described in this thesis is the requirement for matching ages for test and control tissue donors.

4.4.4 Advanced lesion

Longitudinal clinical studies have confirmed that most gingivitis lesions whether persistent or transient, are not progressive. Only some lesions progress to periodontitis (Lindhe et al., 1975, Page, 1986); however, factors causing this conversion are not understood.

Clinically, periodontitis is defined as chronic inflammation that extends beyond the gingiva to the deeper periodontal structures, producing loss of connective tissue attachment, pocket formation and alveolar bone resorption. The involved tissue may present with the clinical signs of gingivitis, a deepened sulcus or pocket, gingival recession, and possibly tooth mobility and drifting reflecting loss of alveolar support (Figure 5).

When gingivitis progresses to periodontitis the advanced lesion presents histologically. In the advanced lesion, the features of the established lesion persist, plasma cells continue to dominate, with many degenerate plasma cells accumulating in foci. The inflammatory infiltrate extends laterally and apically into the surrounding connective tissues. Continuing proliferation of the epithelium along the root of the tooth is accompanied by loss of tissue attachment from the root surface resulting in the formation of a cleft or pocket (Figure 6). The periodontal pocket is lined by epithelial cells that form a narrow, irregular band of loosely organised epithelial cells, one to ten cells wide with finger-like projections extending into the inflamed subgingival connective tissue (Figure 7). Loss of alveolar bone and periodontal ligament fibres, and disruption of the tissue architecture are features of the advanced lesion.
Figure 5  Advanced periodontitis displaying features of gingivitis with loss of attachment and pocket formation detected by probing. Using a Hu Friedy PC10 colour calibrated probe (3-6-8-10) a periodontal pocket with an 8mm probing depth is located on the mesio-buccal aspect of 15
Figure 6 Diagram comparing the anatomy of tissues that are minimally inflamed or have gingivitis with those affected by periodontitis. In health, the gingival soft tissues are minimally inflamed and attached to the tooth at the junction of the crown and the root. With the accumulation of bacterial plaque in the gingival sulcus, the gingival soft tissues become inflamed, establishing the non-destructive lesion of gingivitis. In some patients, there is a progression of gingivitis to periodontitis, with destruction of the supporting periodontal ligament, loss of alveolar bone, and pocket formation over a period of many years.
Figure 7 Advanced periodontitis biopsy section stained with haematoxylin and eosin displaying an extensive ICI subjacent to OSE and PE and little intact CT. OE= oral epithelium, OSE=oral sulcular epithelium, PE= pocket epithelium, ICI=inflammatory cellular infiltrate
4.5 MODELS FOR DISEASE ACTIVITY

The mechanisms involved in the development and progression of periodontitis are not known. There is wide individual variation in the pattern of destruction, which also varies over time and at different sites within the same individual.

Several models of disease activity have been proposed to account for the inconsistent patterns of disease progression and rate of destruction, manifest clinically as loss of attachment. These models are based on clinical observation. They include continuous progression where loss of attachment occurs at a slow rate over long periods of time or acute episodic bursts with rapid destruction at individual sites (Socransky et al., 1984) or a combination of both models (Jeffcoat and Reddy, 1991, Machtei et al., 1993). The asynchronous burst model postulates that the majority of periodontal destruction occurs during a defined period of activity followed subsequently by an essentially quiescent phase (Socransky et al., 1984).

The assumption that inflammation and loss of attachment are governed by the same biological processes, although not validated, underpins the concept of disease activity which uses serial pocket probing depths to measure loss of attachment, as a reflection of disease activity (Goodson et al., 1982). Indeed, all clinical diagnostic techniques are limited, providing only retrospective data about past disease and are incapable of diagnosing disease activity. For example, the periodontal probe used for determining loss of attachment suffers from the significant limitation of measuring in only one dimension (Watts, 1995). The capacity to identify loss of attachment in more than one dimension is needed for a better understanding of disease pathogenesis and for improved detection and monitoring of periodontal diseases.

No studies of the histopathology of the lesion have been able to support or refute the concept of disease activity. This is not surprising as disease activity relates to clinical changes observed over at least two points in time, whereas studies of the histopathology of the periodontitis lesion are limited by tissue samples taken at one point in time, yet representing a past history of events which are temporally indistinguishable.
4.6 THE STUDY OF PERIODONTITIS

4.6.1 Advances in knowledge and problems confronting the study of periodontitis

Periodontitis is recognised as a multifactorial disease. Therefore, the identification and elucidation of the role of each factor is necessary in order to contribute to an understanding of the aetiology and pathogenesis of this chronic inflammatory disease.

The precise aetiologcal factors have yet to be identified although there are several Gram-negative anaerobic pathogens including, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans* (Zambon, 1996), that have a strong association with adult periodontitis. Furthermore, identification of all disease modifiers, both environmental and genetic or acquired is essential to our understanding of the disease mechanisms involved. This knowledge should lead to a better understanding of susceptibility to disease, severity and rate of disease progression, as well as therapeutic responsiveness.

Considerable progress has been made in our understanding of periodontitis. The extensive literature on periodontitis is based principally on *in vitro* and *in vivo* studies using animal models and humans. A brief summary of some recently recognised key features follows.

As previously stated, the bacteria most strongly associated with periodontitis are *P. gingivalis*, and *A. actinomycetemcomitans*. Other species are probably involved but they act more indirectly than these species. Recent studies *in vitro* indicate that these bacteria are potentially tissue invasive providing an entirely different perspective to our understanding of periodontitis. *P. gingivalis* and *A. actinomycetemcomitans* have the capacity to evade and to modulate host immune processes. Furthermore, the highly organised biofilm supporting these putative pathogens is largely protected from host defences allowing a persistent and evolving presence of microorganisms in close proximity to gingival tissue.

Bacteria are necessary but the action of bacterial products alone is insufficient to account for all of the features of the disease. Host factors are important in determining disease progression and outcome. Periodontitis-involved tissue displays high levels of: LPS; proinflammatory cytokines IL-1, TNF-α, and IL-6; prostaglandins (PGE), especially PGE2; and the matrix metalloproteinases (MMPs). Acquired and
environmental risk factors, for example, tobacco smoking, modify the host response. In addition, there is emerging evidence for a genetic basis for susceptibility to all forms of periodontal disease. Polymorphism in the gene family for IL-1, especially IL-1β, is associated with development of severe periodontitis later in life (Kornman et al., 1997, Engebretson et al., 1999).

The study of cytokines and their source in periodontal diseases has been the focus of much research. The local host response to periodontal pathogens results in the production of inflammatory mediators and cytokines that are thought to play a significant role in the pathogenesis of periodontitis. Microbial substances, such as LPS, can activate macrophages, fibroblasts and keratinocytes to secrete proinflammatory cytokines, including TNF-alpha and IL-1β. It is widely held that either excessive or continuous production of cytokines is responsible for the progression of periodontitis and periodontal tissue destruction. The inflammatory cytokines, IL-1α, IL-1β, IL-6, and IL-8 are present in diseased periodontal tissues, and their unrestricted production appears to play a role in chronic leukocyte recruitment and tissue destruction (Okada and Murakami, 1998, Graves, 1999). Evidence has emerged that the overproduction of IL-1β occurs in certain individuals who are susceptible to severe periodontitis later in life (Kornman et al., 1997, Engebretson et al., 1999). Macrophages have been identified in treated periodontitis tissues as sources of IL-1β (Lo et al., 1999), IL-1α, IL-6, IL-8, and TNF-α (Matsuki et al., 1992).

Although there is an extensive literature on cytokines in periodontal diseases, further research is required to elucidate their roles, particularly as most studies have used extracted cells or treated tissue samples, making it unclear as to how these cytokine profiles relate to the natural pathology.

To develop a better understanding of the pathogenesis of periodontitis further elucidation of the biology of the periodontal tissues is required. For example, little is known about the biology of the pocket epithelium, which functions as a critical interface with the microbial flora of the pocket and as rate limiting barrier against ingress of microbial products into the underlying connective tissue and vasculature. Recently,
studies of the biology of untreated pocket epithelium were undertaken which will be described later in this chapter.

4.6.2 Treated versus untreated tissue in studies of periodontitis

The objective of periodontal therapy is to arrest disease progression and stabilise periodontal attachment levels. This is achieved by physically disrupting the subgingival biofilms by scaling and root planing. Therefore, therapy at disease sites is aimed at reducing aetiological factors below the threshold capable of producing breakdown, thereby allowing repair of the affected area (Pihlstrom and Ammons, 1997).

Histologically, there is reduction of inflammation in the subjacent connective tissue and the formation of a long junctional epithelium, which appears to remain stable as long as the subgingival microbial flora is not re-established. The effect of treatment on the pathological features of pocket epithelium, the underlying vasculature and the capacity to develop reparative granulation tissue is uncertain. Notwithstanding these unknowns, in the majority of studies of human periodontal disease, tissue specimens have been obtained from lesions that have been treated therapeutically in some way. Patterns of change detected in tissues following therapeutic interventions such as scaling or root planing may not reflect the natural history of the disease process accurately. Presumably, the use of manipulated tissue introduces another confounder in a complex multifactorial disease process.

There is a need for re-evaluation of the literature with regard to results derived from the use of treated and pooled treated and untreated periodontal tissues. More recently, attention has focussed on identifying alterations in the levels of inflammatory products present in GCF in treated and untreated tissues. Furthermore, the studies reported next and in Chapters 5 to 8 were designed specifically to address this problem by ensuring that all tissues examined were removed prior to initiation of any treatment.

4.6.3 Recent studies in vivo using untreated periodontitis tissue

Recent studies of the untreated advanced periodontitis lesion have revealed extensive vascular pathology, including alterations in blood vessel morphology with thickening of the basement membrane (Zoellner and Hunter, 1989, Pinchback et al., 1996a), the
accumulation of fragments of basement membrane, persistent foci of degenerate plasma cells (Joachim et al., 1990, Pinchback et al., 1996a) and restricted capacity to develop reparative granulation tissue (Zoellner and Hunter, 1989, Pinchback et al., 1996a). The distribution and functional status of macrophages in relation to these pathological features is unknown.

4.6.3.1. Pocket epithelium studies

The source of the non-keratinizing squamous epithelial cells lining the pocket is not known. It is thought to arise from the junctional epithelium but there is also potential for contribution by developmental remnants (Spouge, 1980). Morphologically, the pocket epithelium appears abnormal, lacking an obvious layered structure and with tenuous intercellular contacts (Pinchback et al., 1996a). The biological basis of this altered differentiation is unknown although an understanding of the regulation of epithelial differentiation in the periodontal pocket is clearly of importance.

The untreated pocket epithelium (PE) has altered characteristics, which have been identified in recent collaborative studies by this writer, N Hunter, and with other researchers at the Institute of Dental Research, Sydney (Hunter et al., 2000a, b). The first study investigated the pathological lining epithelium of destructive periodontitis by analysis of the expression of intermediate filament proteins in biopsies of untreated advanced periodontitis (Hunter et al., 2000a).

Cytokeratins (CK) are a group of intermediate filament proteins that form part of the intracellular cytoskeleton in epithelial cells. These proteins are subdivided into the acidic type 1 keratins (CK10 to CK20) and the neutral-basic type II keratins (CK1 to CK9) that form heterodimers characteristic of the tissue type and the position of cells within the strata of stratified squamous epithelia (Moll et al., 1982).

CK8/18 form a primitive cytokeratin pair characteristic of simple epithelia in the adult and are expressed in many epithelia during embryonic development. CK8/18 are not a feature of stratified squamous epithelia in the adult and it is only in severe dysplasia and neoplasia that the keratinocytes are found to express these proteins (Morgan et al., 1987, Lindberg and Rheinwald, 1990). In our study, CK8/18 was expressed consistently in a distribution pattern confined to the reactive pocket epithelium. The pattern of CK 8/18 expression was complex with two broad
presentations evident. In two thirds of the advanced disease biopsies, the entire pathological lining epithelium was strongly reactive for both CK8 and CK18. In the remainder, the more superficial lining epithelium was mixed with foci of reactive and unreactive cells with the deeper epithelium uniformly reactive. Only occasional highly localised reactivity for the simple keratins (CK 8/18) was found in the lining epithelia of biopsies from minimally inflamed periodontal tissues.

The CK5/14 heteropolymer pair is characteristic of the basal layers of stratified epithelia while CK4/13 is characteristic of suprabasal cells and CKs 1, 2, 10, 11 are typical of cornified envelopes (Moll et al., 1982). CK6, 16 and 17 are characteristic of high turnover or migrating epithelium (Paladini et al., 1996). In our study (Hunter et al., 2000a) the pathological lining epithelium of advanced periodontitis was further characterised by the co-expression in basal layers of CK14, and of CK 13 but not CK 4. Cytokeratin 17 was extremely variable with no clear association between expression pattern and location of the epithelium or disease status. There was no reactivity for CK 10/11 nor of vimentin, the characteristic intermediate filament of mesenchymal cells. The intermediate filament protein profile of the reactive lining epithelium was indistinguishable from the reactive epithelium present in three of five biopsies of periapical granulomas containing hyperplastic epithelium from activation of the developmental remnants of Hertwig's sheath known as the cell rests of Malassez. Our results were interpreted as showing that the reactive lining epithelium of the subgingival pocket in untreated chronic periodontitis continues to express an intermediate filament profile that reflects an origin from developmental epithelium of the enamel organ and cell rests of Malassez. How a primitive epithelium responds to microbial challenge is unknown and yet it assumes importance in the pathogenesis of periodontal diseases.

In the second study, the writer, P Ye and others investigated the structural integrity and functional differentiation of the lining epithelium in relation to inflammatory changes associated with destructive periodontitis (Ye et al., 2000). In the different regions of lining epithelia from clinically healthy gingiva and periodontitis, we compared expression patterns of E-cadherin, which is critical in intercellular adhesion, of proteins associated with gap junction communication channels and of involucrin, which is a key marker of differentiation in stratified epithelia. Filamentous actin (F-
actin), which is important in cell structural integrity, attachment and migration was also examined. Semiquantitative immunohistochemical analysis revealed that in both clinically healthy gingiva and lesions of advanced periodontitis, expression patterns of E-cadherin, involucrin and connexins 26 and 43 were similar, with statistically significant reduction in staining intensity from the external oral epithelium through the gingival sulcus to the junctional epithelium or pocket epithelium, respectively. Furthermore, there was a striking reduction in staining for E-cadherin, involucrin and both connexins in the pathological lining epithelium of the periodontal pocket. These changes were associated with marked alterations of F-actin expression, collectively indicating profound perturbation of epithelial structure. Our data support the concept that the ability of the pathological lining epithelium to function as an effective barrier against the ingress of microbial products into the tissues is severely compromised.

Others have also reported altered expression of adhesion proteins and integrins, and disrupted basement membrane of the PE (Haapasalmi et al., 1995). It appears likely although unproven, that most of these epithelial changes are related to the microflora of the pocket. In this context, the role of macrophages presumably assumes importance in defence and repair of the subjacent tissue environment.

TGF-β is a superfamily of regulatory proteins that include the isoforms, TGF-β1, -β2, and -β3 identified in mammals. TGF-β has three major biological effects: growth inhibition, stimulation of extracellular matrix formation, and immunosuppression.

The dual role of TGF-β in modulating macrophage function is an important concept gaining increasing recognition (Ashcroft, 1999). TGF-β has strong macrophage-deactivating effects as well as exerting macrophage-activating effects (Bogdan and Nathan, 1993). These functions are modulated by the state of differentiation of the cell, the local cytokine environment, as well as the local levels of TGF-β. In general, during the initial stages of inflammation, TGF-β acts locally as a proinflammatory agent by recruiting and activating resting monocytes to express TNF, IL-1 and IL-6 and mediating host defence. As these cells differentiate specific immunosuppressive actions of TGF-β predominate, such as suppression of TNF release at the translational level (Bogdan and Nathan, 1993), leading to resolution of the inflammatory response.
(Ashcroft, 1999). The TGF-β isoforms and the types, affinity, and signaling functions of the TGF receptors I and II, add complexity to the regulation of these effects.

Although regulatory mechanisms of TGF-βs and their receptors in different cell types have been studied extensively, little is known about the contribution of the TGF-βs in inflamed gingival epithelial cells. In a recent study at the Institute of Dental Research this writer, and co-workers (Ye et al., in preparation) investigated the presence of TGF-β₁, -β₂, and -β₃ and their receptors (R) I and II in pocket epithelium of untreated periodontitis using immunohistochemistry. TGF-β₁ and TGF-β RI were not detected either in gingival epithelia from minimally inflamed or periodontitis biopsies. There was over-expression of TGF-β₂, -β₃ and TGF-β RII in the pocket epithelium compared to the minimally inflamed epithelium. Furthermore, expression of TGF-β₁ in macrophages in untreated periodontitis tissue was investigated using dual immunofluorescence and confocal microscopy. Preliminary data suggest that some CD163+ macrophages express TGF-β₁. This finding supports those of Steinsvoll and others (Steinsvoll et al., 1999). Taken together, these findings support a potential immunosuppressive role for the pocket epithelium and macrophages.

4.6.3.2 Vascular-associated leukocyte adhesion molecules

A collaborative study was undertaken by N Hunter, this writer, and others (Hunter et al., 2000b) to compare the distribution of vascular-associated leukocyte adhesion molecules in advanced periodontitis with minimally inflamed gingival tissues. Markers typical of acute inflammatory lesions and comprising P-selectin, E-selectin and PECAM-1 (CD31) were widely expressed with similar numbers of reactive vessels detected in both minimally inflamed tissues and advanced lesions. In relation to the substantial evidence for a protective role of neutrophils in periodontal disease (Katsuragi et al., 1988, Zoellner and Hunter, 1991b), the adhesion molecules expressed in minimally inflamed tissues could be considered to be appropriate for successful defence of the tissues. In contrast, the destructive lesion of advanced periodontitis is characterised by a manifest failure of protective responses and yet the adhesion molecules expressed were comparable to that in minimal inflammation. An unexpected
finding was the marked accumulation of B cells and plasma cells associated with P-selectin and E-selectin reactive vessels in advanced disease. In contrast, there was only trace reactivity for VCAM-1, the adhesion molecule with specificity for mononuclear leukocytes, in both advanced disease and minimally inflamed specimens. How this pattern of expression of adhesion molecules affects the recruitment of monocytes to the site of inflammation has yet to be determined. Dual staining for P-selectin and type IV collagen indicated that many of the reactive vessels were affected by a previously described perivascular deposition of basement membrane components (Zoellner and Hunter, 1989, Pinchback et al., 1996a). This could explain the failure of neutrophil migration but leaves unanswered whether the kinetics of monocyte migration is altered, as well as the paradox of accumulation of plasma cells around these vessels (Hunter et al., 2000b).

4.7 STUDIES OF MACROPHAGES IN PERIODONTITIS

Chronic inflammatory diseases are characterised by three main reactions: vascular, cellular and immune responses. In other chronic inflammatory diseases, and presumably in periodontitis, the multifunctional macrophage plays a significant role in each of these reactions.

Although the pathology of chronic periodontitis has been studied extensively there is a paucity of data relating to the role of macrophages in this disease process. Early studies used morphological criteria to define the inflammatory infiltrate in periodontitis lesions. Accurate identification and quantification of specific cell types was not always possible. Often in the morphological description of pathological processes general terms such as round-cell infiltration, phagocytic cells and mononuclear cells were used. By not defining the specific cells interpretation of the data has been limited.

One histological study of advanced periodontitis in young adults (21 to 28 years) compared diseased tissue with established gingivitis and minimally inflamed, clinically healthy gingival tissue within each subject. It was noted that cells belonging to the monocyte-macrophage lineage were present in low numbers in clinically healthy gingival tissue and only a modest increase was detected in established gingivitis sites.
and in advanced disease (Lindhe et al., 1980). Whether a similar increase occurs in adult periodontitis is not known.

More recently, antibody probes with improved specificity have been used to identify macrophage phenotypes in clinically healthy gingival tissues, in developing experimental gingivitis (Topoll et al., 1989) and in treated periodontitis specimens (Johannessen et al., 1986, Joly et al., 1986, Schlegel-Gomez R et al., 1995). It is however, unclear how these findings relate to macrophage distribution and function in the untreated destructive disease process.

There have been few investigations into the functional status of macrophages in treated periodontitis lesions and only one in untreated tissue examining nonspecific esterase and acid phosphatase activity by macrophages (Charon et al., 1981).

There is a requirement to define the distribution and functional status of macrophages in relation to pathological changes. In the next chapter an investigation of the distribution of phenotypic distinct macrophage populations in untreated periodontitis is described and in the following chapter a study of key markers of functional activation for digestive capacity and antigen presentation in macrophages within the advanced periodontitis lesion are investigated.