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**The effect of oral bacteria on
cytokine and antimicrobial peptide
expression in oral epithelia**

BACTERIAL-EPITHELIAL CELL
INTERACTION) IN THE PERIODONTAL DISEASE.

Thesis submitted by
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Abstract

Periodontal diseases result from a complex interaction between a biofilm containing commensal and periopathogenic bacteria and the host innate and acquired defense systems. The interaction of oral commensal and pathogenic bacteria and their effect on cell behaviour, particularly the synthesis of antibacterial and inflammatory molecules, has been the focus of this project. The messenger RNA (mRNA) and protein expression of human beta-defensin and pro-inflammatory cytokine mRNA in the gingiva of patients suffering from the periodontal diseases was also determined.

Patients suffering periodontal diseases showed increased mRNA expression of human beta-defensins and cytokines compared to controls, however, there was no difference in human beta-defensin protein expression between diseased and control tissue samples.

Further studies were then carried out to determine the effect of oral commensal and periopathogenic bacteria and their surface components on oral epithelial cells (OECs). An oral squamous carcinoma cell line was found to produce IL-8 protein and express mRNA for human beta-defensin 2 (hβD-2), both of which were induced by several oral bacterial cell surface components, including LPS. The stimulatory effect of LPS was subsequently found to involve the LPS receptor, CD14. The presence of toll-like receptor mRNA was also demonstrated and results showed that their expression may be regulated by bacteria associated molecular patterns.

Both live- and heat-killed oral bacterial pathogens, *A. actinomycetemcomitans* and *P. gingivalis* induced production of IL-8 protein and hβD-2 mRNA from OECs. Exposure to the oral commensals *S. sanguis* and *S. gordonii* resulted in a decrease in the production of IL-8 mRNA from OECs, whilst heat-killed *S. sanguis* upregulated hβD-2 mRNA.

A highly invasive strain of *A. actinomycetemcomitans* was shown to adhere to OECs to a greater degree, and also led to a greater induction of hβD-2 mRNA and IL-8 protein compared to a non-invasive strain. Further, isogenic mutants of the oral commensal *S. gordonii* DL1 Challis, deficient in the production of antigen I/II-family proteins SspA and SspB and the fibrillar cell surface proteins CshA and CshB, showed reduced

adhesion to OECs. All strains had comparable effects on IL-8 protein and h β D-2 mRNA expression in OECs.

The results presented in this thesis demonstrate the expression profile of human beta-defensins and cytokines in healthy and diseased gingival tissue. h β D-2 has been shown to be upregulated in oral epithelial cells by a range of oral commensal and pathogenic bacteria and their products. It has also been shown that the invasive nature of oral bacteria may contribute to increased expression of h β D-2 messenger RNA in oral epithelial cells.

The upregulation of h β D-2 mRNA by a wide variety of components, bacterial or otherwise in oral epithelial cells may have therapeutic potential, however further studies would need to be carried out to determine the correlation between mRNA and protein expression of h β D-2.

Declaration

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated. Samples of gingival tissue from patients with localised aggressive periodontitis were collected by Steven Smith (Department of Periodontology, Eastman Dental Hospital, 256 Gray's Inn Road, London, WC1X 8LD). The RNA from the gingival tissue was subsequently extracted and reverse transcribed by Dr. Peter Tabona (Division of Microbial Diseases, Eastman Dental Institute, 256 Gray's Inn Road, London, WC1X 8LD).

Acknowledgements

I would like to thank my supervisors Professor Mike Wilson and Professor Brian Henderson for their continued support, encouragement and guidance throughout my PhD.

Whilst not my supervisors, although they may have felt like it at times, I would also like to thank Dr. Sean Nair, Dr. Simon Whawell and Dr. Peter Tabona for all their technical advice and assistance, in addition to their encouragement and friendship.

I am indebted to Dr. Stephen Poole from the National Institute of Biological Standards and Control for being a generous source of antibodies for the IL-8 ELISAs employed throughout this thesis.

I would also like to thank all my friends and colleagues, both past and present, in the Division of Microbial Disease and the Department of Oral Medicine for their continued help and for being great friends, particularly, Dr. Alison Marshall, Miss Lorna Seville, Dr. Adam Roberts and Dr. Lindsay Sharp.

My special thanks go to Dr. Holli Lancaster, Miss Zoey Jackson, Miss Emma Jaffray, Dr. Jon Pratten and Dr. Derren Ready, whose friendship has been very important to me throughout my PhD.

I would also like to take this opportunity to thank both families, whose support through my many years of studying has been very welcome.

Finally to Will my best friend and soul mate, who has been there every step of the way. His constant support, encouragement and faith in me and of course his invaluable IT skills have seen me through my PhD.

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List of abbreviations

APES	3-amino propyl tri ethoxysilone
BHI	Brain Heart Infusion
Bp	Base Pair
°C	Degrees Celsius
cDNA	Complimentary DNA
CO ₂	Carbon dioxide
CFU	Colony Forming Units
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
EOP	Early Onset Periodontitis
FACS	fluorescence associated cell sorting
FbpA	fibronectin binding protein
FCS	Foetal Calf Serum
G	Gravitational Force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	Hour
hβD-2	Human beta-defensin 2
HSP	heat shock proteins
HRP	horseradish peroxidase
IL-8	Interleukin 8
kb	Kilobase
kDa	Kilodalton
KGM	keratinocyte growth medium
L	Litre
LBP	LPS-binding protein
LAP	Localised aggressive periodontitis
LJP	Localised juvenile periodontitis
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
µg	Microgram
µl	Microlitre
min	Minute
mg	Milligram
ml	Millilitre
mM	Micro-Molar
NaCl	sodium chloride
NF-κB	Nuclear factor κB
OECs	Oral epithelial cells
PAMPs	pathogen-associated molecular-patterns
PB	Polymixin B
PBMC	peripheral blood mononuclear cells

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SAG	salivary agglutinin glycoprotein
SAM	surface-associated material
sec	Second
SLPI	secretory leukocyte protease inhibitor
TAP	Tracheal antimicrobial peptide
TECs	tracheal epithelial cells
TLR	Toll-like receptors
TBE	Tris borate EDTA
TSB	trypticase soy broth
UV	Ultra Violet
YE	Yeast extract

Chapter 1

Introduction

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It is estimated that 10-15% of the world's population has periodontitis. This chronic inflammatory and destructive disease is believed to be due to alterations in the normal interaction between the oral tissues and the resident microbiota. A number of different oral commensal bacteria are implicated in the pathology of periodontal diseases, in some way the interaction of these bacteria, in the form of a biofilm, with host tissues drives a process of periodontal inflammation and tissue destruction (Landi *et al.*, 1997).

The mucosal epithelium provides a physical barrier to microbes, but it is now clear that it also has an active role in the host innate defence system. Epithelial cells, one of the primary cell types associated with periodontal and oral mucosal health (Darveau *et al.*, 1997, Tonetti and Mombelli, 1997), are in constant contact with microbes and their products.

In certain bacterial infections, the ability of a pathogenic bacterium to adhere to epithelial cells is the starting point in the disease process. The possibility that adhesion may lead to invasion of periodontal cells by oral bacteria and play a role in the pathogenesis of the periodontal diseases has also been suggested (Meyer *et al.*, 1997) and there is increasing evidence that many bacteria can invade many cell types in culture (Sandros 1993, Meyer *et al.*, 1997a, Ahmed *et al.*, 2001, Cossart and Sansonetti, 2004). Epithelial cells actively respond to bacteria in an interactive manner. For example, they secrete IL-8 and other chemokines and cytokines to alert various cell types and to attract neutrophils. They also produce natural antimicrobial peptides in response to bacterial plaque. These antimicrobial peptides are part of the innate immune system, a complex set of responses that keeps microbial invaders in check and maintains the indigenous microbiota of the healthy periodontal pocket (Weinberg *et al.*, 1998).

1.1 The structure of the epithelial barrier

1.1.1 The periodontium

The term periodontium defines the tissues that surround and support the tooth. These include the gingiva, the periodontal ligament, the cementum, and the alveolar bone (Figure 1.1). The nerve and vascular supplies of the involved tissues are also essential to the normal functioning of the periodontal tissues. In health, the structure and function of the tissue components of the periodontium are interdependent, with their dynamic biological adaptation and renewal processes maintaining a harmonious relationship.

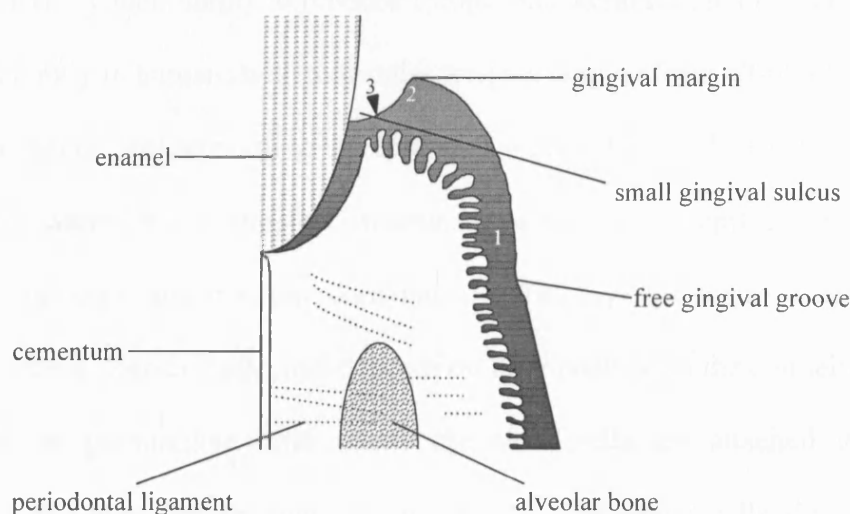


Figure 1.1 Diagrammatic representation of the periodontium and gingiva: (1) gingival oral epithelium; (2) gingival sulcular epithelium; (3) gingival junctional epithelium

1.1.1.1 Gingiva

The gingiva functions as a protective barrier against microorganisms found in dental plaque. The mucosal epithelial cells of the gingiva play an important part in innate immune defence by sensing signals from the external environment and generating molecules to affect the growth, development and function of other cells, whilst maintaining the balance between health and disease.

The gingival epithelium is a stratified squamous epithelium surrounding the tooth and forming an attachment to the tooth surface. Based on architectural characteristics specific for the areas related to the teeth, the gingival epithelium can be divided into the gingival oral epithelium, the gingival sulcular epithelium and the gingival junctional epithelium.

1.1.1.2 The gingival oral epithelium

The gingival oral epithelium extends from the gingival margin to the mucogingival junction facing the oral cavity, thus covering the clinically visible part of the free gingiva and the attached gingiva. The vast majority of cells in the epithelium are keratinocytes, characterised by their ability to produce cytoplasmic keratin filaments. The keratins form a complex family in humans with molecular weights ranging from 40-68 kDa. Their function is to give mechanical strength to the epithelial sheet (Moll *et al.*, 1982). There are several layers that comprise the classical structure of a keratinised epithelium: stratum basale, stratum spinosum and stratum corneum (keratin layer), (figure 1.2). The shape of keratinocytes is characteristic, and depends on their position in the epithelium. The stratum basale is the germinative layer where the basal cells are attached to the basement membrane and here they are columnar or cuboidal. The largest cells, the spinous cells, are found in the middle of the epithelium, the stratum spinosum, and here they are polygonal. Those cells found in the superficial layer are flattened with their long axis parallel to the epithelial surface.

Under normal conditions, the oral epithelium contains cells whose morphology and function are different to the keratinocytes. These include melanocytes, dendritic cells that produce melanin to protect the epithelium from actinic radiation and another non-keratin-producing intraepithelial cell, the Langerhan's cell. Langerhan's cells are dendritic antigen-presenting cells present within the epithelium of the skin and mucosal. These cells

probably play an important part in the early host response to microbial antigens at the gingival margin. (Barrett *et al.*, 1996).

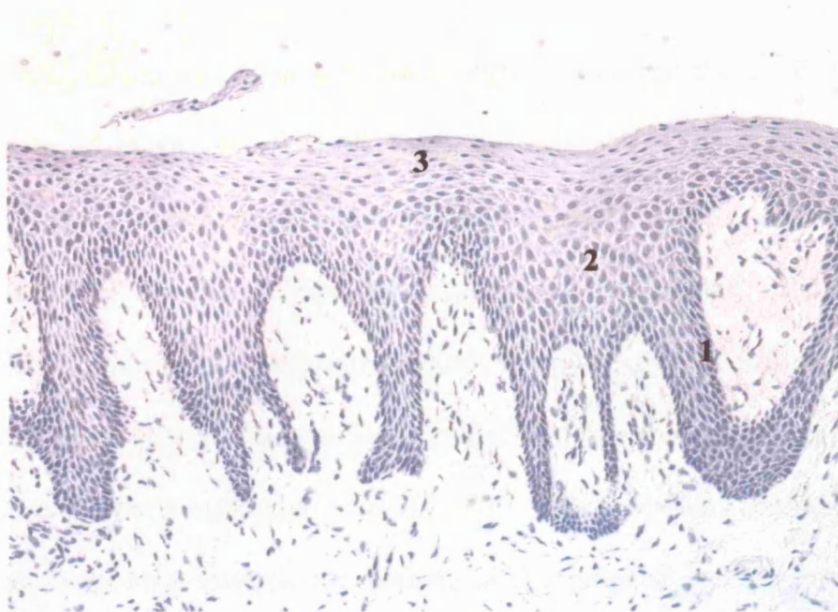


Figure 1.2 Parakeratinized gingival oral epithelium and its stratification: (1) basal cell layer (stratum basale); (2) spinous cell layers (stratum spinosum); (3) parakeratinized cell layer (stratum corneum).

1.1.1.3 The gingival sulcular epithelium

The gingival sulcular epithelium extends from the oral epithelium into the gingival sulcus facing the tooth and is of limited distribution in health. The sulcular epithelium is similar to the oral epithelium apart from its lack of a stratum corneum. It has been speculated that this lack of a keratinised layer renders the gingival sulcus particularly susceptible to microorganisms (Hassell, 1993).

1.1.1.4 The gingival junctional epithelium

The gingival junctional epithelium plays a very important part in periodontal health and disease, since it forms part of the attachment between the tooth and the gingiva (figure 1.1). This specific epithelium has adapted to its role for adherence to the tooth surface

making it different, in several aspects, from the other forms of epithelia. When healthy, the gingival junctional epithelium is thinner, has a specific cytokeratin profile and has an even interface with the connective tissue. In the junctional epithelium, the suprabasal cells are flattened with their long axis parallel to the tooth surface. Between the epithelial cells there are few intercellular junctions and spaces thereby reducing the adhesion between cells compared to other gingival epithelia. These spaces facilitate the diffusion of tissue fluids from the connective tissues, through the epithelia, into the gingival sulcus. Conversely, the orientation of the suprabasal cells, the unkeratinised surface and the intercellular spaces also permit the passage of bacterial products from the gingival sulcus to the connective tissue (Listgarten, 1986). The junctional epithelium, like the oral and sulcular epithelium contains Langerhan's cells (Barrett *et al.*, 1996). The junctional epithelium also contains neutrophils migrating towards the bottom of the gingival sulcus, even in periodontal health. These cells may, therefore, be important in preserving the integrity of the healthy periodontium.

1.2 The indigenous oral microbiota

Bacteria are found in the oral cavity from shortly after birth until death. They colonise the soft tissues of the mouth including the soft palate, the cheeks, gingiva and the tongue. Once teeth have erupted they also colonise them below and above the gingival margin. Any individual may typically harbour 500-1000 or more different species.

1.2.1 Oral biofilms

In the oral cavity, an important aspect of microbial ecology is the accumulation of dental plaque composed of microbes, their waste products, host cells and nutrients in a highly specialized environment known as a biofilm. A biofilm has been defined as “a functional consortium of microorganisms” that is normally contained within a polymer matrix (Costerton *et al.*, 1994). The biofilm structure has many advantages for the microbes contained within it including: increased protection from antimicrobial agents, from phagocytosis by host immune cells and an increase in concentration of nutrients and the potential for genetic exchange. Recent reviews have described the importance of dental plaque as a microbial biofilm (Rosan and Lamont, 2000, Sbordone and Bortolaia, 2003, Marsh, 2004).

1.2.2 The formation of dental plaque

The formation of dental plaque starts with the acquired pellicle, which consists of various salivary components that coat the tooth surface, such as mucins and α -amylase (Kolenbrander and London 1993, Rosan and Lamont, 2000, Li *et al.*, 2003). The primary colonisers of the acquired pellicle are usually streptococci, principally the sanguis group. These initial colonisers appear to have adhesins with multifunctional roles at their cell surface (section 1.3.1). *Streptococcus parasanguis* has a lipoprotein which is involved in a transport system but also binds bacterial carbohydrate receptors, proteins in human saliva

and fibrin (Fenno *et al.*, 1995). Streptococci not only bind to other microbes (coaggregation) but also to each other (aggregation) (Jenkinson and Lamont, 1997, Rosan and Lamont, 2000).

Streptococcal aggregation is followed by coaggregation with other Gram-positive bacteria such as actinomycetes, haemophili and veillonellae (Kolenbrander, 1993, Rosan and Lamont, 2000). As the bacterial population grows and becomes more complex there is a shift from mainly Gram-positive to Gram-negative bacteria. Early colonisers coaggregate with each other and with fusobacteria. The late colonisers of the oral cavity then, almost exclusively, adhere to *Fusobacterium* spp. (Kolenbrander, 1993, Kolenbrander, 2000, Sbordon and Bortolaia, 2003, Foster and Kolenbrander, 2004). *Fusobacterium* spp. are found in high numbers in both healthy and diseased individuals (Kolenbrander and London, 1993, Kolenbrander, 2000). Their ubiquitous nature is due to a diverse metabolic repertoire: they can ferment amino acids and use the resulting energy to transport the sugars available in human saliva (Kolenbrander, 1993). The late colonisers, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans* and *Treponema* species, are all periodontal pathogens, which depend on fusobacteria. It has been suggested that fusobacteria act as a bridge between early and late colonising bacteria (Kolenbrander and London, 1993, Kolenbrander, 2000, Foster and Kolenbrander 2004). A diagrammatic representation of the patterns of bacterial co-aggregation in human dental plaque can be seen in figure 1.3.

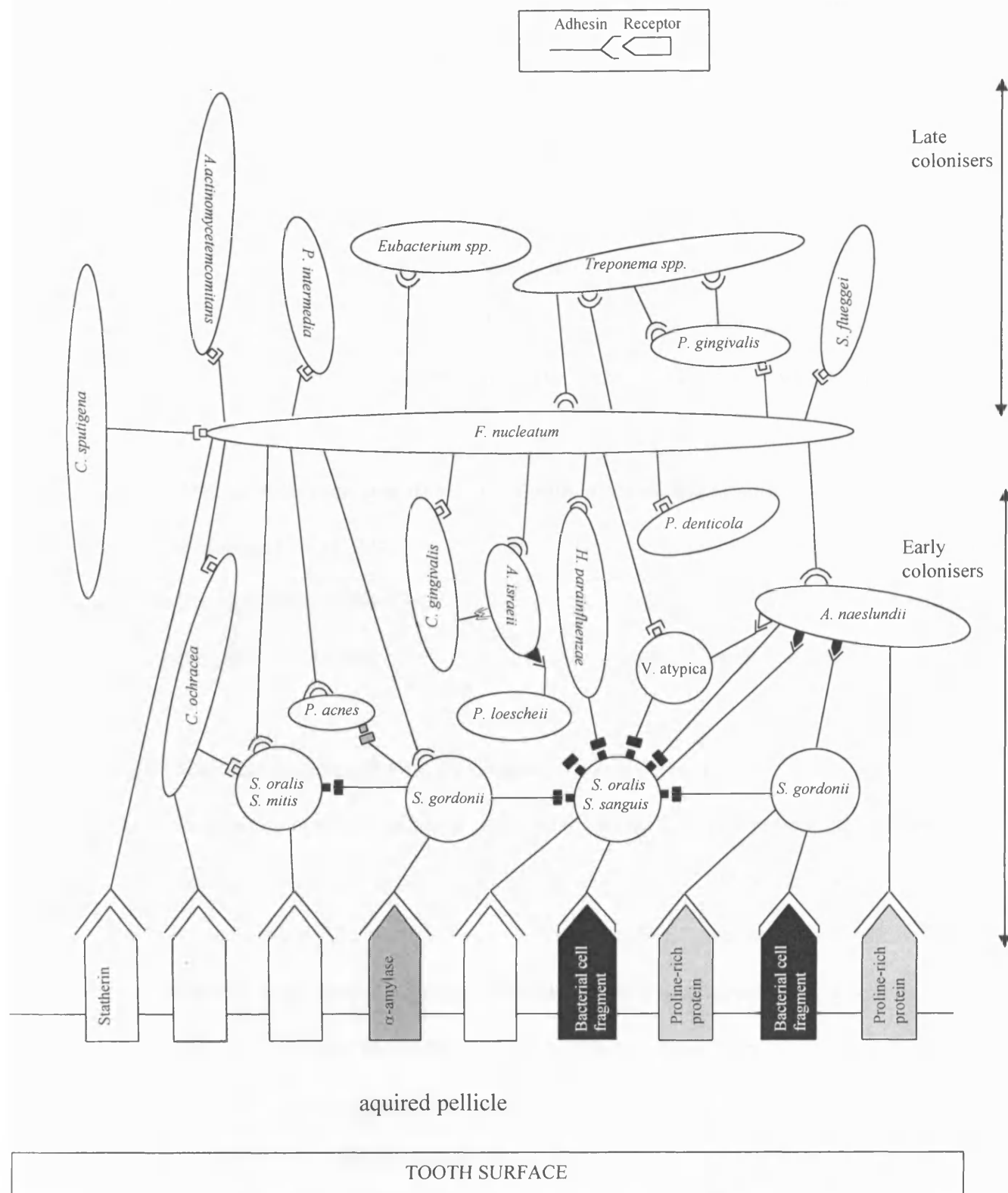


Figure 1.3 Diagrammatic representation of the patterns of bacterial co-aggregation in human dental plaque (adapted from Kolenbrander and London, 1993)

1.3 The periodontal diseases

The periodontal diseases are a series of infections of the periodontal tissues which can lead to eventual loss of teeth due to the destruction of associated alveolar bone and periodontal ligament (Page, 2002, Graves and Cochran, 2003). The diseases represent the result of a complex interaction between the biofilm of commensal bacteria, the periopathogenic bacteria, connective tissue cells and host innate and acquired immunity. Periodontitis progresses periodically, alternating between comparatively short periods of rapid tissue destruction and longer episodes of disease latency (Goodson *et al.*, 1982, Socransky *et al.*, 1984).

In the 1999 classification system for periodontal diseases and conditions, over 40 different gingival diseases were listed (Armitage, 1999). In addition, 7 major categories of destructive periodontal diseases were listed including: chronic periodontitis, localised aggressive periodontitis and generalised aggressive periodontitis (Armitage, 1999).

1.3.1 Bacteria associated with gingival health and disease

The bacteria associated with gingival health and various forms of periodontal disease have been extensively studied over the past 20 years (Tanner *et al.*, 1996, Ximenez-Fyvie *et al.*, 2000, Rudiger *et al.*, 2002). Clinically healthy gingival sites have a bacterial pattern consistent with that described in relatively immature supragingival plaque (section 1.2.2 and figure 1.3). Certain members of the microbiota are common in bacterial plaque whether the samples are obtained from supra- or subgingival sites in diseased or healthy subjects. This core group of organisms are typically built around the *Actinomyces*, but members of the genera *Streptococcus*, *Veillonella*, *Capnocytophaga*, *Eikenella*, *Leptotrichia* and *Neisseria* also appear to play prominent roles (Slots, 1979, Moore and Moore 1994, Ximenez-Fyvie *et al.*, 2000).

Studies of bacteria cultured from experimental gingivitis show a shift from a streptococcal dominated plaque to one in which *Actinomyces* spp. predominate. The overall composition of the microflora associated with gingivitis is different and more diverse than that found in health, also there is an increase in plaque mass (Rudiger *et al.*, 2002).

Increased proportions of Gram-negative anaerobic rods and *Prevotella* and *Porphyromonas* spp. have been found compared to healthy sites in plaque-associated gingivitis (Rudiger *et al.*, 2002, Lovegrove, 2004). More extensive studies have found that levels of Gram-negative rods, including *Eikenella corrodens*, *Fusobacterium nucleatum* and *Capnocytophaga* species are also increased in plaque samples taken from gingival sites (reviewed in Moore and Moore, 1994).

Chronic periodontitis is the most common form of advanced periodontal disease that affects the general population and has a worldwide prevalence of 10-15% (Papapanou, 1999). Bacterial plaque accumulation leads to a chronic inflammation of the gingiva. The gingiva then becomes detached and migrates down the tooth surface. This causes a distinct gap between the tooth surface and gingiva, creating a pocket that is caused by the migration of the junctional epithelium along the root. Eventually the underlying alveolar bone is resorbed, leading to possible loss of the tooth (Nair *et al.*, 1996, Page, 2002, Graves and Cochran, 2003) (for diagram of the periodontium see figure 1.1).

The inflammatory response to plaque in chronic adult periodontitis contributes to the tissue destruction, with the release of cytokines including various interleukins, tumor necrosis factor alpha (TNF- α) (Okada *et al.*, 1998), oxygen radicals and lysosomal enzymes released during phagocytosis, from infiltrating neutrophils. This considerable response by the host immune system can lead to even more tissue damage (Kinane and Lappin, 2001).

Bacterial species associated with chronic adult periodontitis are more commonly Gram-negative obligate anaerobes or capnophiles, which include, *A. actinomycetemcomitans*,

Prevotella intermedia, *Porphyromonas gingivalis*, *Eikenella corrodens* and *F. nucleatum* (reviewed in Moore and Moore, 1994). A recent study, using molecular techniques, has shown that increased proportions of *P. gingivalis*, *Tannerella forsythensis*, and species of *Prevotella*, *Fusobacterium*, *Campylobacter* and *Treponema* were detected subgingivally in periodontitis subjects (Ximénez-Fyvie, 2000). *P. gingivalis*, *T. forsythensis* and *Treponema denticola* were significantly more prevalent in both supra- and subgingival plaque samples from periodontitis patients (Ximénez-Fyvie, 2000). Periodontal pathogens such as those implicated in chronic adult periodontitis meet several criteria. High numbers are found in periodontal lesions but not in healthy sites (Slots and Genco 1984, Slots and Listgarten 1988, Moore and Moore 1994), the organism elicits high antibody titers in patient's serum, gingival crevicular fluid and saliva (Ebersole *et al.*, 1991, Kinane *et al.*, 1999, Papapanou *et al.*, 2004). These microorganisms also have an extensive array of virulence factors and cause disease in animal models (Klausen 1991, Weinberg and Bral, 1999). *A. actinomycetemcomitans* and *P. gingivalis* in particular meet these criteria (Cutler *et al.*, 1995, Wilson and Henderson, 1995, Fives-Taylor *et al.*, 1999, Henderson *et al.*, 2003).

Periodontal diseases are among the most frequent diseases affecting children and adolescents. These include gingivitis, localised aggressive periodontitis (LAP) and generalised aggressive periodontitis, previously known as early onset periodontitis (EOP), which included generalised or localised juvenile (LJP), rapidly progressive and juvenile periodontitis (Listgarten, 1986a, Armitage, 1999, Tonetti and Mombelli, 1999). LAP exhibits rapid destruction of periodontal tissue in adolescents, with a distinct pattern of alveolar bone loss (Genco *et al.*, 1986, Williams, 1990). The disease affects 0.1-2.3% of juveniles and adolescents, with a higher prevalence in blacks and other minorities (Loe and Brown 1991).

The most frequently detected microorganisms in LAP include *A. actinomycetemcomitans*, *Capnocytophaga* sp., *E. corrodens*, *P. intermedia*, and motile anaerobic rods, such as *Campylobacter rectus*. Gram-positive isolates were mostly streptococci, actinomycetes, and peptostreptococci (Tonetti and Mombelli, 1999). Generalised early-onset periodontitis and rapidly progressive periodontitis have been frequently associated with the detection of *P. gingivalis*, *T. forsythensis* and *A. actinomycetemcomitans* (Vandesteen *et al.*, 1984, van Steenberghe *et al.*, 1993a, Lopez *et al.*, 1995).

A. actinomycetemcomitans has been repeatedly implicated in LAP, with a range of studies listing *A. actinomycetemcomitans* as the causative agent responsible for the pathogenesis of the disease (Mandell 1984, Asikainen 1986, Mandell *et al.*, 1987 Lopez *et al.*, 1995).

Large numbers of *A. actinomycetemcomitans* are recurrently isolated from patients, along with fewer numbers of *Capnocytophaga* spp., *E. corrodens*, *F. nucleatum*, *Bacteroides capillus* and *Eubacterium brachy* (Moore *et al.*, 1985, Moore, 1987). Many studies have reported frequencies around 90% for *A. actinomycetemcomitans* in periodontal lesions of LAP patients (Slots *et al.*, 1980, Zambon *et al.*, 1983, Mandell 1984, Asikainen 1986). In some studies it was possible to demonstrate elevated levels of *A. actinomycetemcomitans* in sites showing evidence of recent or ongoing periodontal tissue destruction (Haffajee *et al.*, 1984, Mandell 1984, Mandell *et al.*, 1987). Investigations have also repeatedly reported significantly elevated levels of serum antibodies to *A. actinomycetemcomitans* in LAP patients (Listgarten *et al.*, 1981, Altman *et al.*, 1982, Sandholm *et al.*, 1987). LAP patients also locally produced antibodies against *A. actinomycetemcomitans* at diseased sites (Ebersole *et al.*, 1985, Tew *et al.*, 1985). The view that *A. actinomycetemcomitans* has a specific role in periodontal disease is a contentious one. Several reports have not confirmed the association between *A. actinomycetemcomitans* and LAP: *A. actinomycetemcomitans* was not detected in any affected subjects studied (Okuda *et al.*, 1984, Vandesteen *et al.*, 1984, Han *et al.*, 1991). In other studies it was not possible to

reproduce the previously reported frequencies of *A. actinomycetemcomitans* in the range of 90% (Gunsolley *et al.*, 1990, Kim *et al.*, 1992, Lopez *et al.*, 1995). In another study high levels and proportions were found for *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *C. rectus*, *T. denticola* and *A. actinomycetemcomitans*, however, was not significantly associated with EOP (generalised, localised or incidental) (Albandar *et al.*, 1997).

1.3.1.1 *Porphyromonas gingivalis*

Porphyromonas gingivalis is an anaerobic, Gram-negative, non-motile short rod. *P. gingivalis* can be cultured from the gingival sulcus, tongue, buccal mucosa and tonsillar area in humans (Van Steenberghe *et al.*, 1993). *P. gingivalis* has been shown to be increased in sites with periodontitis and significantly lower or non-detectable in sites with gingival health or plaque-associated gingivitis (White and Mayrand, 1981, Slots and Listgarten, 1988).

This microorganism has been shown to have an extensive range of virulence factors, including various enzymes (proteases, collagenase, gelatinase, peptidase and hyaluronidase) (Slots and Genco, 1984, Potempa *et al.*, 2003), haemagglutinin activity (Okuda *et al.*, 1986), cytokine induction (Darveau *et al.*, 1998), and adherence factors (fimbriae, vesicles etc.) that promote colonisation and invasion of host cells (Weinberg *et al.*, 1997, Meyer *et al.*, 1997).

1.3.1.2 *Actinobacillus actinomycetemcomitans*

Actinobacillus actinomycetemcomitans is a Gram-negative, non-spore forming, non-motile, facultatively anaerobic coccobacillus. It was first described by Klinger in 1912 and named *Bacterium actinomycetem-comitans* because it was frequently found with *Actinomyces israelii* in cervicofacial actinomycotic lesions. As mentioned previously, *A. actinomycetemcomitans* is an important pathogen in severe and recurrent forms of

periodontal disease. It has a prevalence of 90% in LAP and 30-50% in chronic adult periodontitis (Asikainen and Alaluusua, 1993).

A. actinomycetemcomitans possesses numerous virulence factors, reviewed in Wilson and Henderson 1995, Fives-Taylor 1999 and Henderson *et al.*, 2002. Known virulence determinants include a leukotoxin (Lai *et al.*, 1981 and Kolodrubetz *et al.*, 1989), adhesiveness, mediated by fimbriae and vesicles (Meyer and Fives-Taylor, 1993 Fives-Taylor *et al.*, 1995), a collagenase (Robertson *et al.*, 1982), a chemotactic inhibitor (Van Dyke *et al.*, 1982), a cytotoxin (Slots, 1982), molecules which cause bone resorption; an endotoxin (Kiley and Holt, 1980) and GroEL (Kirby *et al.*, 1995), immunosuppressive factors (Shenker *et al.*, 1990), Fc binding proteins (Tolo and Helgeland, 1991) and a bacteriocin (Stevens *et al.*, 1987).

1.4 Bacteria-host interactions in the oral cavity

Bacterial colonisation of gingival tissue is a critical process in the pathogenesis of periodontal disease leading to its penetration and destruction. Many oral bacteria exhibit adhesins, which enable them to bind to other bacteria, epithelial cells and to extracellular matrix proteins (Chen and Duncan, 2004, Hamada *et al.*, 2004, Mintz, 2004, Ruhl *et al.*, 2004). Bacterial cell binding to host cells, in addition to aiding colonisation, may be a prelude to intracellular invasion and modulation of the immune response. These events may also be precursors to development of disease. Adhesion and invasion may therefore be mechanisms by which oral bacteria such as streptococci can influence the biological activities of the host cell (Vernier, 1996, Al-Okla *et al.*, 1999).

In order to reach and cause destruction to underlying tissue, periodontopathogens or their products or components must be able to pass through the epithelial cell barrier. As mentioned previously, adherence to host oral tissues leading to invasion are key virulence factors for many periodontopathogens. In particular, adhesins of

A. actinomycetemcomitans and *P. gingivalis* enable them to bind to other oral bacteria, to teeth, to extracellular matrix and to epithelial cells, the first step in their invasion process (Meyer *et al.*, 1997, Fives-Taylor 1999, Yilmaz *et al.*, 2002, Amornchat *et al.*, 2003).

1.4.1 Streptococcal adhesion molecules

Salivary components include secretory products of the salivary glands, bacterial products such as glucan polysaccharides, dietary components (which may include lectins and other molecules), serum products which originate as an exudate in gingival crevicular fluid; and other compounds entering whole saliva by gastric and respiratory reflux (Gibbons, 1984). These salivary components coat the tooth surface, buccal and lingual epithelial cells, and all other biological and non-biological (eg. prosthetic) surfaces within the mouth. Streptococcal adhesion results primarily from initial binding of cells to these salivary components. A large number of distinct adhesins have been described. It is apparent that sanguis group streptococci are endowed with multiple adhesins for salivary molecules (Jenkinson and Lamont, 1997). The streptococcal salivary adhesins that have been described in the most detail are the Lral family of polypeptides and the antigen I/II family of polypeptides. Reviews have provided extensive information on both of these families (Whittaker *et al.*, 1996, Jenkinson and Demuth, 1997).

Streptococci also possess multiple adhesins for human cells; reviewed by Jenkinson and Lamont, 1997, which further substantiates their status as successful colonisers. The extent to which there is direct contact between bacteria and the host cell is uncertain. *In vivo*, cells would be coated with mucus, salivary secretions and matrix proteins. However, to date, although *in vitro* experiments have demonstrated adhesion of bacteria to epithelial cells, this has been in the absence of such salivary components (Wang and Stinson, 1994, Han *et al.*, 2000). Bacterial cell binding to host cells, in addition to aiding colonisation, may be a prelude to intracellular invasion and modulation of the immune response. These events

may also be precursors to the development of disease. Adhesion and invasion may, therefore, be mechanisms by which oral bacteria such as streptococci can influence the biological activity of the host cell.

The antigen I/II family of polypeptides are produced by virtually all streptococci indigenous to the oral cavity and are highly conserved. The structure, function and immunogenicity of the streptococcal antigen I/II polypeptides have been reviewed by Jenkinson and Demuth, 1997.

Streptococcus gordonii is known to express, on the cell surface, two antigen I/II polypeptides designated SspA and SspB (formerly Ssp 5) that are products of tandemly arranged chromosomal genes (Demuth *et al.*, 1996). These polypeptides, designated Ssp5 (in *S. gordonii* M5) and SspA (in *S. gordonii* DL1), are implicated in the binding of bacterial cells to salivary agglutinin glycoprotein (SAG) (Demuth *et al.*, 1990, Jenkinson *et al.*, 1993) a reaction that is inhibited by sialic acid (Demuth *et al.*, 1990).

The SspA polypeptide has been shown to be involved in binding of streptococci to *Actinomyces naeslundii* (Jenkinson *et al.*, 1993) an organism found closely associated with *S. gordonii* and *S. oralis* in dental plaque (Kolenbrander and London, 1993, Rosan and Lamont, 2000). Evidence suggests that the antigen I/II polypeptides are also involved in the interactions of *S. gordonii* with *P. gingivalis* (Lamont *et al.*, 1994) and *Candida albicans* (Holmes *et al.*, 1996). Expression of the *S. gordonii sspB* gene in *Enterococcus faecalis* (Demuth *et al.*, 1989) conferred on enterococcal cells the ability to bind *P. gingivalis* and *C. albicans* (Lamont *et al.*, 1994, Holmes *et al.*, 1996).

Two antigenically related high-molecular-mass proteins designated CshA and CshB are also produced by *S. gordonii* DL1 (challis) and are encoded by genes at distinct chromosomal loci (McNab *et al.*, 1994). CshA and CshB bind to actinomyces cell surface molecules (McNab *et al.*, 1992, 1994). Isogenic CshA mutants are deficient in binding

fibronectin (McNab *et al.*, 1996) and to *A. naeslundii* and *S. oralis* (McNab *et al.*, 1994 and 1996). Insertional mutation in *cshA* has also been shown to cause reduced cell surface hydrophobicity (McNab and Jenkinson 1992). Recent evidence suggests that the CshA polypeptide is the structural and functional component of *S. gordonii* adhesive fibrils and they provide the molecular basis for past correlations of surface fibril production, cell surface hydrophobicity, and adhesion in species of oral “sanguis-like” streptococci (McNab *et al.*, 1999). Mutations in the *cshB* gene have less effect on hydrophobicity and coaggregation. Both CshA and CshB were required to confer *S. gordonii* with the ability to colonise the murine oral cavity (McNab *et al.*, 1994).

In addition to their interaction with bacterial cells, antigen I/II polypeptides and cell-surface rhamnose-glucose polymers may enable oral streptococci (including mutans group streptococci, *S. anginosus*, *S. constellatus*, *S. gordonii*, *S. intermedius*, *S. milleri*, *S. oralis* and *S. salvarius*) to stimulate the production of IL-8 in mammalian cells (Vernier *et al.*, 1996).

1.4.1.1 Streptococcal adhesion to host cells

We know that streptococci indigenous to the oral cavity are endowed with multiple adhesins for salivary molecules, fibronectin, other oral bacteria and *C. albicans* (Demuth *et al.*, 1990, Jenkinson *et al.*, 1993, Lamont *et al.*, 1994, Holmes *et al.*, 1996, McNab *et al.*, 1996). Streptococci also possess multiple adhesins for human cells (Jenkinson and Lamont, 1997). Adhesion of group A and B streptococci to epithelial cells may be a crucial determinant in the pathogenesis of streptococcal infections. Lipoteichoic acid (LTA), composed of a repeating poly glycerol phosphate and covalently attached fatty acids, is present on the streptococcal cell surface and has long been implicated in adhesion of streptococci to epithelial cells (Simpson *et al.*, 1980). Whilst evidence is convincing that LTA is involved in adhesion of Group A streptococci to buccal and pharyngeal cells and to

HEp-2 (laryngeal) tissue culture cells, (Wang and Stinson, 1994, Perez-Casal *et al.*, 1995 Okada *et al.*, 1995) it appears to play little role in Group B streptococcal adhesion (Tamura *et al.*, 1994).

A major receptor for group A streptococci is fibronectin, deposited on the surfaces of human buccal cells (Courtney *et al.*, 1996). Adhesion of bacteria to buccal cells is thought to occur by the sequential binding of LTA and one (or more) of the fibronectin-binding adhesins present on the streptococcal cell surface (Courtney *et al.*, 1992). *S. gordonii* CshA mutants are deficient in binding fibronectin, a major receptor for Group A streptococci binding to buccal epithelial cell surfaces (McNab *et al.*, 1996).

Upon gaining access to the bloodstream, many streptococci (particularly oral species) are able to infect the heart valves and endocardium, causing endocarditis (Weinberger *et al.*, 1990). *S. sanguis* is capable of inducing platelet aggregation through a number of surface components, a trypsin-sensitive class I adhesin mediates initial attachment of the bacteria to the platelets. Aggregation is then induced by platelet aggregation-associated protein (PAAP) an antigenitically distinct class II adhesin (Herzberg, 1996, Kerrigan *et al.*, 2002). Oral bacteria which have gained access to the circulation as a result of periodontal disease, leading to the subsequent interaction of bacteria with platelets, may therefore play an important role in cardiovascular diseases (Kerrigan *et al.*, 2002).

Not much information exists on the interactions between oral streptococci and oral epithelial cells. However, studies have shown that *S. salivarius* binds human buccal epithelial cells through an antigen C fibrillar glycoprotein (Handley, 1990).

Further research shows that insertional inactivation of the *sspA* gene in *S. gordonii* DL1 results in reduced binding of cells to SAG, human erythrocytes and the oral bacterium *A. naeslundii* (Demuth *et al.*, 1996). Greater reduction in streptococcal cell adhesion to SAG and to two strains of *A. naeslundii* were observed when both *sspA* and *sspB* were

inactivated, suggesting both polypeptides are involved in adhesion of *S. gordonii* to human and bacterial receptors (Demuth *et al.*, 1996).

1.4.2 Periodontal pathogen adhesion and invasion of host cells

To date, there are several reports of putative oral pathogens, associated with periodontitis, which invade oral epithelial and fibroblasts of the gingiva. These include *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *F. nucleatum* (Meyer *et al.*, 1991, Lamont *et al.*, 1992, Duncan *et al.*, 1993, Sreenivason *et al.*, 1993, Dorn *et al.*, 1998, Han *et al.*, 2000).

A. actinomycetemcomitans and *P. gingivalis* are the two organisms on which most periodontal research has focused and where invasion of gingival tissue *in vivo* has been clearly demonstrated (Saglie *et al.*, 1986, Saglie *et al.*, 1988, Rudney *et al.*, 2001). In addition, the invasion of epithelial cells *in vitro* by these two organisms has also been demonstrated (Lamont *et al.*, 1992, Fives-Taylor *et al.*, 1994, Fives-Taylor, 1999, Yilmaz, 2002).

Invasion of *P. gingivalis* has been demonstrated in a human oral epithelial cell line, KB cells (Duncan *et al.*, 1993), human pocket epithelium (Sandros *et al.*, 1993), human buccal epithelial cells (Rudney *et al.*, 2001) and primary cultures of gingival epithelial cells (Lamont *et al.*, 1995). *P. gingivalis* binding to epithelial cells is thought to be mediated by fimbriae (Lamont *et al.*, 1995, Weinberg *et al.*, 1997). More recently, evidence suggests that fimbriae bind to cytokeratins on the surface of epithelial cells (Sojar *et al.*, 2002). Upon binding, *P. gingivalis* triggers an active process, which results in internalisation. Using inhibitors of bacterial and epithelial cell functions, Lamont *et al.*, 1995, determined that entry requires both *P. gingivalis* and host cell energy metabolism. However, only bacterial protein, DNA and RNA synthesis are necessary for invasion. After

internalisation, *P. gingivalis* can be observed free in the cytoplasm, where it can multiply and persist (Madianos *et al.*, 1996, Houalet-Jeanne *et al.*, 2001).

A strain of *P. intermedia* has been shown to invade an oral epithelial cell line by Dorn *et al.*, 1998, and type C fimbriae and cytoskeleton rearrangement were required for invasion. Other evidence from Han *et al.*, 2000 showed another strain of *P. intermedia* to have no invasive properties. However, *F. nucleatum* was shown to adhere to and invade epithelial cells. Invasion appeared to occur via a “zipping” mechanism and required the involvement of actin, microtubules, signal transduction, protein synthesis and energy metabolism of the epithelial cell, as well as protein synthesis by *F. nucleatum* (Han *et al.*, 2000).

Most investigations into tissue attachment and invasion by oral bacteria have focused on *A. actinomycetemcomitans*. *In vitro* models have demonstrated that *A. actinomycetemcomitans* enters epithelial cell lines, primary gingival epithelial cells and buccal epithelial cells (Meyer *et al.*, 1991 and 1996, Fives-Taylor *et al.*, 1995, Rudney *et al.*, 2001).

A. actinomycetemcomitans invasion is a highly dynamic complex process, involving the attachment of organisms to the host cell with initiation of some form of signalling, through binding to a receptor, entry in a vacuole, escape from the vacuole, rapid multiplication, intracellular spread, exit from the cell and cell-to-cell spread, the process can be seen in figure 1.4 (Meyer *et al.*, 1996).

Most *A. actinomycetemcomitans* strains that have been tested adhere strongly to epithelial cells (Meyer and Fives-Taylor 1994). Cell surface components, which mediate adherence, include fimbriae, extracellular amorphous material and extracellular vesicles (Meyer and Fives-Taylor 1993, 1994). Studies reported by Meyer *et al.*, 1997, suggest the primary receptor for *A. actinomycetemcomitans* invasion is the transferrin receptor (Meyer *et al.*, 1997). Invasion was inhibited by human and mouse transferrin, while ovotransferrin (a molecule of similar size) which is unable to bind to the human transferrin receptor had no

effect on invasion (Meyer *et al.*, 1997). Upon entering the host cell, the internalised bacteria are surrounded by an actin halo. A clinical isolate, *A. actinomycetemcomitans* SUNY 465, has been shown to enter epithelial cells by an actin-dependant mechanism (Brissette and Fives-Taylor 1999). *A. actinomycetemcomitans* is then taken up in a host-derived membrane bound vacuole, which lyses shortly thereafter and enters the cytoplasm (Sreenivasan 1993). Phospholipase C, a molecule implicated in vacuole lysis by some enteric pathogens, has been suggested as the lysis agent (Meyer and Fives-Taylor 1997). After escape from the vacuole into the cytoplasm, *A. actinomycetemcomitans* transits through the cell to neighbouring cells via bacteria-induced protrusions, which appear to be extensions of the host cell membrane. If a neighbouring cell is not present, bacteria are released from the cell by rudimentary protrusions. Microtubules have been strongly implicated in intra- and intercellular spread of *A. actinomycetemcomitans* and its release into the extracellular environment (Meyer *et al.*, 1996 and 1999). A characteristic of the intracellular existence of *A. actinomycetemcomitans* is its rapid rate of replication. The organism is a fastidious, slow growing organism, however, within the host cell cytoplasm it appears to double much more rapidly, about every 20 minutes. This rapid intracellular replication appears to be linked to the microtubule-dependant host cell spread (Meyer *et al.*, 1996). It has been hypothesised that the *in vitro* observations of the dynamic process of *A. actinomycetemcomitans* invasion of epithelial cells are involved in its ability to spread to the gingival and connective tissue and initiate the destruction associated with periodontal disease (Fives-Taylor *et al.*, 1999).

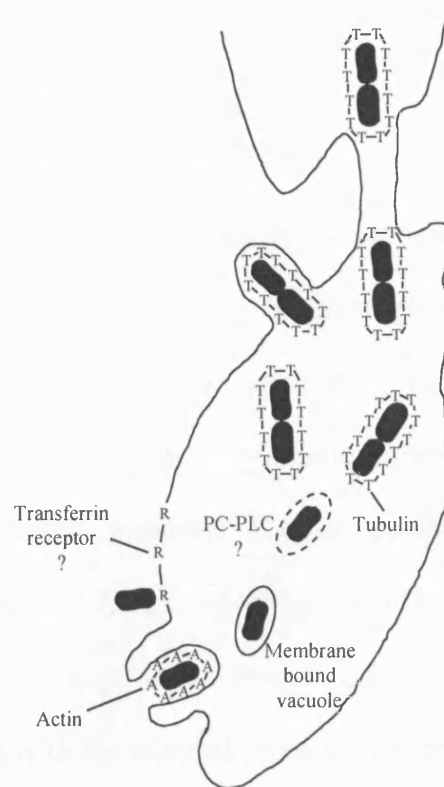


Figure 1.4 Schematic representation of invasion of epithelial cells by *Actinobacillus actinomycetemcomitans*. R, receptor; T, tubulin; A, actin; PC-PLC, phosphatidylcholine-preferring phospholipase C. Adapted from Meyer *et al.*, 1997.

1.5 Host responses in periodontal disease

Host responses, primarily directed to defending the host against infection, but often tissue damaging, along with the direct destructive effects of bacteria, result in the tissue destruction seen in periodontal disease. In order for the host to maintain homeostasis in the oral cavity, three distinct but interrelated immune response systems contribute to controlling the microbial colonisation. These are: (i) the salivary (ii) gingival tissue (local) and (iii) the circulating (systemic) immune systems. Cells mediating immunity include lymphocytes and phagocytes, these cells recognise antigens on the invading microorganisms and internalise and degrade them. During an immune response, the

antigen is recognised by specific lymphocytes which lead to their activation, in the subsequent effector phase the source of the antigen is eliminated by the immune response coordinated by the lymphocyte. On subsequent encounters with a particular antigen the immune system is able to mount a more effective response. This is due to essential features of the adaptive immune response, specificity and memory.

Innate immunity is mediated by the circulating/tissue phagocytic cells: monocytes, macrophages and polymorphonuclear neutrophils. These cells bind to microorganisms, internalise, and then destroy them. They bind to a variety of microorganisms since they have a non-specific recognition system. The neutrophilic polymorphonuclear leukocyte, or neutrophil, is a phagocyte, which is especially important in defending against oral bacteria, particularly those causing periodontal disease (Genco 1992). Phagocytes provide our first line of defence along with the external physical barriers such as skin, the gastrointestinal tract and the urogenital tract.

The local host response to oral bacteria, the recruitment of leukocytes and the subsequent release of inflammatory mediators and cytokines, appear to play crucial roles in the pathogenesis of periodontal diseases. Although cytokines are produced by locally infiltrated immunocompetent cells, such as T-cells and monocytes at the diseased sites, cell types that normally compose the tissue – such as fibroblasts, epithelial cells and endothelial cells – are also involved in cytokine production during the inflammatory response (reviewed in Okada and Murakami, 1998).

1.5.1 Cytokines

Cytokines are intracellular messengers whose biological activity is dependant on binding to selected high affinity receptors on target cells and generating particular patterns of intracellular signalling, resulting in selected gene transcription (Saklatvala *et al.*, 2003). Included under the term cytokine are interleukins, interferons, growth factors, cytotoxic

factors, activating and inhibitory factors, colony stimulating factors and chemokines. Cytokines play important roles in tissue homeostasis and also in the pathogenesis of many infectious diseases. Research on the biological activities in the normal periodontium and the pathogenesis of periodontal disease has clarified the involvement of various inflammatory cytokines at these sites (Wilson *et al.*, 1996).

1.5.1.1 Proinflammatory cytokines

An inflammatory cytokine is defined as a cytokine that is induced during the inflammatory response and is closely associated with its onset and/or progression. Interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8) and TNF- α are generally classed as inflammatory cytokines. The roles of the inflammatory cytokines in periodontal disease have been the subject of review (Gemmell *et al.*, 1997, Okada and Murakami, 1998).

IL-1 β and TNF- α have a key role in the initiation, regulation and perpetuation of innate responses in the periodontium (Birkedal-Hansen 1993, Alexander and Damoulis 1994, Gemmell *et al.*, 1997). A variety of cell types have been shown to secrete IL-1 β and TNF- α in response to periodontal bacteria (Dennison and Van Dyke, 1997). Tissue IL-1 β levels are higher in the gingiva of patients with periodontal disease than in tissue from healthy subjects and higher in active periodontitis sites than stable sites (Honig *et al.*, 1989, Stashenko, 1991). TNF- α from monocytes and macrophages has synergistic effects with IL-1 cytokines, although TNF- α is somewhat less potent than IL-1 (Okada and Murakami, 1998). IL-1 β is a potent stimulator of connective tissue destruction, including breakdown of periodontal ligament and alveolar bone (Tatakis 1993, Kornman *et al.*, 1997)

IL-6 is known to be a bone resorptive cytokine produced by many cell types, including macrophages, T cells, endothelial and epithelial cells and fibroblasts (Agarwal *et al.*, 1995, Okada *et al.*, 1997, Irwin and Myrillas, 1998).

1.5.1.1.1 Interleukin 8

IL-8 is a potent chemotactic factor for leukocytes which participates in the initiation and maintenance of inflammatory reactions (Bickel, 1993, Roebuck, 1999, Mukaida, 2000).

IL-8 attracts and activates neutrophils, the first immune cells recruited to infected sites (Baggiolini *et al.*, 1989). IL-8 is secreted by a variety of cells, including monocytes, fibroblasts, lymphocytes, epithelial and endothelial cells. A substantial amount of information exists regarding inflammatory mediators and cytokines produced by gingival fibroblasts, including IL-8, in response to bacterial challenge in periodontal disease (Takada *et al.*, 1991, Tamura *et al.*, 1992, Dongari-Bagtzoglou and Ebersole 1996).

More recently, the cytokine or mediator profiles of gingival epithelial cells following their interaction with periodontopathogens have been investigated (Huang *et al.*, 1998, Uchida *et al.*, 2001, Yumoto *et al.*, 1999). IL-8 has also been shown to be induced in stimulated gingival epithelial cells by IL-1 α and IL-1 β (Sfakianakis *et al.*, 2001).

1.6 Host immune responses to oral bacteria

As previously mentioned, interactions between bacteria and host cells lead to the release of one or more cytokines. The resulting cytokine network (Wilson *et al.*, 1998) demonstrates the host's attempt to deal with the particular invading organism and constitutes an important part of the host innate immune response.

The ability of bacteria and their components, or their activities (microbial metabolism) to induce cytokine release from numerous host cells has long been regarded as an important aspect of bacterial virulence (Henderson and Wilson, 1995).

The detection of constitutive and conserved products of microbial metabolism seems to be the strategy of which innate immunity is based. As many as 15 classes of bacterial surface components or secretory products are known to stimulate cytokine release (Henderson and Wilson 1996 and Wilson *et al.*, 1996). This wide range of potent cytokine-inducing

proteins, carbohydrates and lipids includes lipopolysaccharide (LPS), lipoproteins, peptidoglycan, lipoteichoic acid (LTA) and fimbrial proteins. It has been suggested that this group of molecules be recognised as modulins, a separate class of virulence factors based on their ability to modulate the behaviour of cells due to the induction of cytokine synthesis (Henderson and Wilson, 1995).

1.6.1 Bacterial modulation of the immune response

Several studies have focused on the effects of whole bacteria, periodontopathogens in particular, on host cells such as gingival fibroblasts and epithelial cells. Many have concentrated on the inflammatory cytokines, IL-1, IL-6, IL-8, and TNF- α . Previously, IL-1 β , IL-6 and IL-8 expression by gingival fibroblasts stimulated with periodontopathogenic bacteria has been thoroughly investigated (Takada *et al.*, 1991 and Dongari-Bagtzoglou and Ebersole 1996).

The production of the chemokine IL-8 and of IL-6 has been shown to be induced in human oral epithelial cells by *F. nucleatum*, *A. actinomycetemcomitans* and *E. corrodens* (Huang *et al.*, 1998, Yumoto *et al.*, 1999, Han *et al.*, 2000, Uchida *et al.*, 2001).

Whilst *P. gingivalis* components, including LPS, have been shown to induce IL-1 β , IL-6, IL-8 and TNF- α from gingival fibroblasts (Imatani *et al.*, 2001), studies by Darveau *et al.*, 1998 showed that *P. gingivalis* strongly inhibited IL-8 accumulation from gingival epithelial cells. Not only did *P. gingivalis* fail to elicit the accumulation of the chemokine but it also inhibited IL-8 accumulation in response to other bacteria such as *F. nucleatum* and *E. corrodens* (Darveau *et al.*, 1998).

Viable and killed *P. gingivalis* are capable of inducing various pro-inflammatory cytokines (IL-1 β , IL-6 and IL-8) from human gingival fibroblasts, however strain differences in cytokine induction have been noted, and the expression of a trypsin-like protease activity was found to be related to decreased extracellular levels of IL-6 and IL-8 (Steffen *et al.*,

2000). *P. gingivalis* supernatants, but not those from *A. actinomycetemcomitans* have been shown to hydrolyse IL-1 β , IL-6 and IL-1ra, and the hydrolysate from the *P. gingivalis* supernatant treated IL-1 β was found to be unable to stimulate the production of IL-6 from human gingival fibroblasts - showing that it had lost biological activity (Fletcher *et al.*, 1997). Studies have also demonstrated that biofilm-grown *P. gingivalis* can also degrade IL-1 β , IL-6 and IL-1ra (Fletcher *et al.*, 1998). IL-8 has been shown to be degraded by *P. gingivalis* proteases (Zhang *et al.*, 1999).

In addition to the induction of IL-8 from oral epithelial cells, *A. actinomycetemcomitans* also stimulates the production of IL-1 α and IL-1 β , it has been suggested that IL-1 mediates the expression of IL-8 through an autocrine and/or intracrine mechanism (Sfakianakis *et al.*, 2001).

When exposed to live and heat-killed *A. actinomycetemcomitans*, gingival epithelial cells show an increase in IL-1 β and IL-8 mRNA, however, the levels when exposed to heat-killed *A. actinomycetemcomitans* were less than those exposed to live *A. actinomycetemcomitans*. These findings suggest that some heat-sensitive components on the *A. actinomycetemcomitans* surface interact with human gingival epithelial cells (HGEC) to induce cytokine mRNA expression (Uchida *et al.*, 2001).

It has been well documented that many human epithelial cells, including intestinal and cervical cells, secrete IL-8 in response to bacterial entry (Eckmann *et al.*, 1993, Jung *et al.*, 1995, Rieder *et al.*, 1997). Studies have shown that whilst invasive bacteria are able to stimulate the release of IL-8, non-invasive bacteria are not (Eckmann *et al.*, 1993, Jung *et al.*, 1995). Also, the observation that direct contact or adhesion of bacteria to epithelial cells is required for cytokine stimulation has been made with both intestinal and oral bacteria (Rieder *et al.*, 1997, Aihara *et al.*, 1997, Yumoto *et al.*, 1999, Han *et al.*, 2000). These data suggest that epithelial cells serve as an early signalling system to host immune and inflammatory cells in the underlying mucosa following bacterial entry. As mentioned

previously, *A. actinomycetemcomitans* can invade and adhere to epithelial cell, and these capabilities have been suggested to contribute to increased cytokine induction (Huang *et al.*, 1998).

1.6.2 Bacterial factors which modulate the immune response

1.6.2.1 Lipopolysaccharide

LPS is an essential component of the cell wall of Gram-negative bacteria. Together with phospholipids and membrane bound proteins it is a constituent of the outer cell membrane. LPS consists of three structural elements: a hydrophobic component, called lipid A, which serves to anchor the molecule into the membrane, a core oligosaccharide containing a characteristic sugar acid, 2-keto-3-deoxyoctulonic acid (KDO) and a heptose and a hydrophilic O-polysaccharide chain that consists of repetitive subunits which make polysaccharides extending out from the bacteria (Erridge *et al.*, 2002, Raetz and Whitfield, 2002, Caroff and Karibian, 2003). Endotoxins are mitogenic for B-cells and function as polyclonal B-cell activators (Peavy *et al.*, 1970), they also mediate the activation of macrophages and proliferation and cytokine production from T-cells (Ulmer *et al.*, 2000). They also act as physiological stimuli for the synthesis of pro-inflammatory cytokines such as TNF- α , IL-1, IL-6 and IL-8 and non-protein mediators (Agarwal *et al.*, 1995, Alexander and Rietschel, 2001).

The induction of the expression of cytokines by LPS is believed to be a crucial event in the activation of the immune system. The lipid A component of LPS is assumed to be responsible for the induction of expression of cytokines (Morrison and Ryan, 1987), which is potentiated by KDO (a sugar which binds the polysaccharide core to lipid A) and the polysaccharide (Haefner-Cavaillon *et al.*, 1989). Mammalian receptors for LPS have been identified in the last decade (Lee *et al.*, 1993, Juan *et al.*, 1995, Palsson-McDermott and O'Neill, 2004). The most important of these is the glycerophosphatidylinositol-linked

protein CD14 (described in section 1.6.3.1). However, CD14 is not by itself capable of initiating a transmembrane activation signal. Recently the newly documented toll-like receptor (TLR) family have been implicated in LPS-signalling (see section 1.6.3.2). Studies of LPS from *E. coli* and *Salmonella* spp. have shown a crucial role for TLR4 in LPS-mediated signalling (Du *et al.*, 1999). It has been suggested that the shape of lipid A determines the interaction of LPS with TLRs (Netea *et al.*, 2002).

The LPS of many oral pathogens have been implicated in the initiation and development of periodontal diseases (Williams 1990, Cutler *et al.*, 1995). The differential expression of IL-1 β , TNF- α , IL-6 and IL-8 in human monocytes in response to LPS from different oral microbes has been investigated (Agarwal *et al.*, 1995). Monocytes from the same subject when activated with LPS from *P. gingivalis*, *A. actinomycetemcomitans* and *E. corrodens* expressed quantitatively different levels of mRNA and proteins for all 4 cytokines.

A given LPS induces either high or low expression of a range of cytokines, indicating that the expression of these pro-inflammatory cytokines may be regulated by a single or cluster of genes (Agarwal *et al.*, 1995). *P. gingivalis* LPS enhances the production of inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α in gingival fibroblasts (Wang *et al.*, 1998).

1.6.2.2 Surface associated material (SAM)

Saline extraction of bacteria releases surface-associated material (SAM), a complex mixture of proteins and carbohydrates with potent biological actions on isolated bone and various mammalian cell populations (Henderson and Wilson, 1996). Using mechanisms dependent on IL-1 and/or TNF- α synthesis, the SAM from a number of oral bacteria, such as *P. gingivalis* and *A. actinomycetemcomitans*, are able to stimulate bone breakdown *in vitro* (Wilson *et al.*, 1985 Meghji *et al.*, 1994, Wilson and Henderson 1995). SAM from *A. actinomycetemcomitans*, *E. corrodens*, *P. gingivalis*, *P. intermedia* and *Campylobacter*

rectus have been found to be capable of stimulating the release of several pro-inflammatory cytokines (IL-1, TNF- α , IL-6 and IL-8) from various human cell populations including monocytes, gingival fibroblasts, peripheral blood mononuclear cells (PBMC) and neutrophils (Reddi *et al.*, 1995 and Reddi *et al.*, 1996b). It has also been found that SAM from *A. actinomycetemcomitans* can, in contrast to *E. coli* LPS, directly induce the synthesis of IL-6 in an IL-1 and TNF- α independent manner (Reddi *et al.*, 1996a).

1.6.2.3 GroEL

Heat-shock proteins (HSPs), which are expressed constitutively in all cells, are essential for several important cellular processes, such as protein folding, protection of proteins from denaturation or aggregation, and facilitation of protein transport through membrane channels (Hartl, 1996).

Rapid destruction of alveolar bone is characteristic of chronic periodontitis and the molecular chaperones have the capacity to induce breakdown of bone and connective tissue. *E. coli* cpn60 (GroEL), but not that of *M. tuberculosis* or *Mycobacterium leprae*, is a potent stimulator of murine bone resorption *in vitro* (Kirby *et al.*, 1995) and such resorption can be inhibited by neutralising the activity of IL-1 with IL-1 receptor agonist (IL-1ra) (Nair *et al.*, 1999). It has been reported that GroEL-like proteins belonging to the hsp60 family can be expressed by periodontopathic bacteria such as *P. gingivalis* (Maeda *et al.*, 1994) and *A. actinomycetemcomitans* (Nakano *et al.*, 1995). *A. actinomycetemcomitans* GroEL has been reported to act as a potent bone-resorbing factor in a murine calvarial resorption assay (Kirby *et al.*, 1995).

From an immunological point of view, HSPs have attracted increasing interest. Bacterial HSPs, in particular, are highly immunogenic molecules able to activate a large number of T-cells (Zugel and Kaufmann 1999). Also, the recognition of hsp60 and hsp70 by antibodies and T-cells has been implicated in a variety of autoimmune and inflammatory

conditions (Kiessling *et al.*, 1991). Srivastava and colleagues, (1998) discovered that HSPs can act as carriers of antigenic peptides derived from tumor cells and virus-infected cells (Srivastava *et al.*, 1998).

Bacterial HSPs such as those from *Mycobacterium* spp. and *E. coli* have been reported to stimulate human monocytes to produce the inflammatory cytokines IL-1 β , IL-6, IL-8 and TNF- α (Freidland *et al.*, 1993, Zhang *et al.*, 1993, Peetermans *et al.*, 1994, Retzlaff *et al.*, 1994, Tabona *et al.*, 1998 Lewthwaite *et al.*, 2001).

E. coli GroES (the 10 kilodalton (kDa) hsp) and GroEL have also been shown to stimulate the release of IL-8 and granulocyte macrophage-colony stimulating factor (GM-CSF) from endothelial cells and also the upregulation of expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1 (Galdiero *et al.*, 1997). This suggests an important role in regulating the expression of cytokines involved in the activation of leukocytes and endothelial cells seen in the initiation of the inflammatory process that accompanies infections with microbial pathogens.

Recent reports have show that CD14, TLR2 and TLR4 signalling are important pathways in mediating the activation of macrophages in response to human hsp60 (Kol *et al.*, 2000 Ohashi *et al.*, 2000 Ueki *et al.*, 2002). The receptors, which mediate human cell response to bacterial HSPs, have yet to be elucidated.

1.6.2.4 Lipoteichoic acid (LTA)

Lipoteichoic acids (LTAs) are amphiphiles, which consist of two parts. One is a polyglycerophosphate, the other is a glycolipid moiety that anchors to the Gram-positive bacterial cytoplasmic membrane by hydrophobic interactions in a manner similar to the interactions between the lipid A of LPS and the Gram-negative bacterial outer membrane.

LTAs are thought to be the counterparts of the LPS of Gram-negative bacteria (see section 1.6.2.1).

Many of the clinical features observed during Gram-positive sepsis have been related to the potent inflammatory properties of LTA (Ginsburg, 2002). LTA triggers the release from neutrophils and macrophages of reactive oxygen and nitrogen species, acid hydrolases, highly cationic proteinases, bactericidal cationic peptides, growth factors and cytotoxic cytokines, which may act in synergy to amplify cell damage (Ginsburg 2002). LTA therefore shares with LPS many of its pathogenic properties. In animal studies, LTA has been shown to induce periodontal lesions (Bab *et al.*, 1979).

It has been demonstrated that LTAs from various Gram-positive bacteria, including oral streptococci, enhanced the production of hepatocyte growth/scatter factor (enhances the motility of epithelial cells) in human gingival fibroblasts and that human gingival epithelial cells produced IL-1 upon stimulation with LPS (Sugiyama *et al.*, 1996). IL-1 α and LTA synergistically induce HGF/SF in human gingival fibroblasts in culture. Therefore, gingival fibroblasts stimulated with *S. sanguis* LTA in the presence of induced IL-1 α may produce a larger amount of HGF/SF, which in turn stimulates gingival epithelial cells to proliferate and migrate to protect periodontal tissues against bacterial invasion (Sugiyama *et al.*, 1996). Studies over the previous decade show that like LPS, LTA induces activation of monocyte/macrophages, inducing stimulation of several cytokines including IL-1 β , TNF- α , IL-6, IL-8 and IL-12 (Bhakdi *et al.*, 1991, Mattsson *et al.*, 1993, Standiford *et al.*, 1994).

1.6.3 Host cell receptors

LPS, lipoproteins, peptidoglycan and LTAs are all molecules made by bacteria but not by eukaryotic cells. Therefore these products can be viewed as molecular signatures of microbial invaders, and their recognition by the innate immune system can signal the presence of infection (Janeway, 1992). Because the targets of innate immune recognition are conserved molecular patterns, they are called pathogen-associated molecular-patterns (PAMPs). The receptors of the innate immune system that recognise PAMPs are called pattern-recognition receptors (PRR) (Janeway 1992).

Various PRRs that are expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids are utilised by the innate immune system, these include CD14 and the human toll-like receptors.

1.6.3.1 CD14

CD14 is a 55kDa glycerophosphatidylinositol (GPI)-anchored protein expressed primarily on the surfaces of monocyte/macrophages and neutrophils and functions principally as the receptor for a complex of LPS (Wright *et al.*, 1990). A soluble form of CD14 (sCD14) lacking the glycerophosphatidylinositol anchor is also present in serum (Wright *et al.*, 1990, Ulevitch and Tobias 1995, Wright 1995). Engagement of LPS with CD14 reportedly induces the transient activation of several protein kinases such as protein kinase C, protein tyrosine kinases and mitogen-activated protein kinases (Weinstein *et al.*, 1992, Shapira *et al.*, 1994). Although CD14 is known to bind LPS, it is a GPI-anchored protein and thus lacks a membrane-bound and intracellular domain, so cannot send signals into the cell. This suggests that another receptor is activating these intracellular signalling pathways. Recently, human gingival fibroblasts have been shown to express CD14 on their cell surface (Hiraoka *et al.*, 1998, Sugawara *et al.*, 1998, Wang *et al.*, 1998). Studies also

demonstrated that high-CD14-expressing fibroblasts secrete IL-8 in response to *P. gingivalis* LPS and LTA and that this is mediated by CD14 (Wang *et al.*, 2001, Sugawara *et al.*, 1999, Watanabe *et al.*, 1996).

Human endothelial cells and colonic epithelial cells have been shown not to express CD14 on their membrane. For activation of these two cell types sCD14 and LBP are required (Pugin *et al.*, 1993). Primary gingival epithelial cells and KB cells are devoid of membrane CD14, as determined by flow cytometry, and showed no enhanced production of IL-8 upon stimulation by LPS, LTA and peptidoglycan even in the presence of serum (Uehara *et al.*, 2001). Since CD14 is unable to send signals into the cell, a genuine receptor of LPS that does send signals into the cells is assumed to exist. Several recent reports have focused on the toll-like receptors, which have been shown recently to recognise bacterial cell wall components (Takeuchi *et al.*, 1999b).

1.6.3.2 Toll-like receptors

The Toll protein controls dorsoventral patterning during the embryonic development of *Drosophila* (Hashimoto *et al.*, 1988). Activation through Toll has been found to induce translocation of Dorsal, the *Drosophila* homologue of NF- κ B and production of antifungal peptides (Lemaitre *et al.*, 1996, Tauszig *et al.*, 2000).

Toll is a type 1 transmembrane receptor with an extracellular domain containing leucine-rich repeats (LRR) and a cytoplasmic domain similar to that of mammalian IL-1 receptor (Gay and Keith, 1991). Human Toll-like receptors (TLR) are membrane bound PRRs homologous to *Drosophila* Toll protein. The extracellular parts of the TLR are quite divergent in their structure, the cytoplasmic signalling domains are conserved and show homologies with other human TLRs as well as with the intracellular domains of IL-1r, IL-18r and the intracellular adaptor protein MyD88 (Rock *et al.*, 1998). Ten TLRs have been described (Rock *et al.*, 1998, Takeuchi *et al.*, 1999a, Du *et al.*, 2000). For TLR2, TLR4

and TLR9 a role in recognition of components of bacterial lipoproteins, of LPS and of bacterial DNA respectively, has been documented (Hemmi *et al.*, 2000, Aderem and Ulevitch 2000).

TLR2 has been shown to be involved in the recognition of a broad range of microbial proteins including: peptidoglycan from Gram-positive bacteria (Takeuchi *et al.*, 1999b, Schwander *et al.*, 1999), bacterial lipoproteins (Takeuchi *et al.*, 2000, Brightbill *et al.*, 1999) and mycobacterial lipoarabinomannan (Underhill *et al.*, 1999). Kirschning and colleagues showed that overexpression of TLR2 could confer responsiveness to LPS in human embryonic kidney 293 cells but this was dependent on the presence of sCD14 in serum. They also found that expression of CD14 synergistically enhanced LPS signal transmission through TLR2 (Kirschning *et al.*, 1998).

It was Takeuchi *et al.*, (1999b) who showed that TLR2 deficient mouse macrophages were hyporesponsive to several Gram-positive bacterial cell walls as well as to *Staphylococcus aureus* peptidoglycan. In addition, TLR2 functions as a receptor for atypical LPS produced by *P. gingivalis*, which is structurally different from other Gram-negative LPS (Hirschfeld *et al.*, 2001). A recent report described that LTA-induced NF- κ B activation in 293 cells is also mediated by TLR2 (Schwander *et al.*, 1999). However, Takeuchi *et al.*, 1999b demonstrated that the responsiveness to LTA was not impaired in TLR2 deficient mouse macrophages.

The broad range of ligands recognised by TLR2 is explained, in part, by cooperation between TLR2 and at least two other TLRs: TLR1 and TLR6. The formation of heterodimers between TLR2 and either TLR1 or TLR6 dictates the specificity of ligand recognition (Ozinsky *et al.*, 2000, Takeuchi *et al.*, 2001). TLR2, for example, cooperates with TLR6 for the recognition of mycoplasmal macrophage-activating lipoprotein 2kDa (MALP-2) (Takeuchi *et al.*, 2001). Human TLR4 was the first characterised mammalian

Toll receptor (Medzhitov *et al.*, 1997). TLR4 is expressed by a variety of cell types, most significantly in the cells of the immune system, including macrophages and dendritic cells. TLR4 functions as the signal-transducing receptor for LPS. Positional cloning of the *Lps* gene in the LPS-non-responsive C3H/HeJ mouse led to this discovery and was confirmed in *Tlr4* knockout mice (Poltorak *et al.*, 1998, Qureshi *et al.*, 1999). Recognition of LPS by TLR4 is complex and requires several accessory molecules. LPS firstly binds to the serum protein LBP (LPS-binding protein) which functions by transferring LPS monomers to CD14 (Wright 1999). Another component of the LPS receptor complex is myeloid differential protein-2 (MD-2). MD-2 is a small protein that lacks a transmembrane region and is expressed on the cell surface in association with TLR4. Although its precise function is not known, MD-2 appears to be required for LPS recognition of TLR4 (Schromm *et al.*, 2001). Thus the LPS receptor complex appears to require CD14, TLR4 and MD-2 for efficient function (Schromm *et al.*, 2001).

In addition to LPS, TLR4 is involved in the recognition of several other ligands, including LTA. Takeuchi and colleagues (1999) showed that TLR4-deficient mouse macrophages lacked response to LTA.

The identification and functional characterisation of TLRs in mammals have brought understanding of the innate immune system to a new level. The role of TLRs in host defence is so fundamental; it is likely their function affects most aspects of the mammalian immune system (Medzhitov 2001).

1.7 Innate immune system in the protection of mucosal surfaces

Mucosal surfaces are protected by both adaptive and innate immune systems. Physical barrier function was formerly believed to play the major role in mucosal protection against bacteria. Recently this view has been challenged by the expanding array of specialised molecules that possess antimicrobial activity (Tomee *et al.*, 1998, Brogden *et al.*, 2003a,

Cunliffe and Mahida, 2004). The fluids lining the mucosal surfaces contain multiple antimicrobial factors, including lysozyme, lactoferrin, secretory leukocyte protease inhibitor and several small antimicrobial peptides known as β -defensins. Epithelial expression of antimicrobial peptides is of particular interest as many pathogens adhere to epithelial surfaces leading to invasion of the host cells. These peptides consist of constitutive and inducible forms, potentiating this barrier function in terms of an inflammatory response.

1.7.1 Secretory leukocyte protease inhibitor (SLPI)

Human secretory leukocyte protease inhibitor (SLPI) is an 11.7 kDa cysteine-rich protein and is an epithelial cell product found in saliva, seminal fluid, and cervical, nasal and bronchial mucus (Hiemstra, 2002, Sallenave, 2002).

Human SLPI is a potent inhibitor of serine proteases; it exhibits inhibitory activity against neutrophil elastase, chymotrypsin, trypsin and cathepsin G (Thompson and Ohlsson 1986, Stetler *et al.*, 1986). Until recently it had been thought that the only function of SLPI was in the protection of mucosal surfaces from degradation by proteases during inflammation (Thompson and Ohlsson 1986). However, SLPI has also been shown to display broad-spectrum bactericidal activity (Hiemstra *et al.*, 1996, Singh *et al.*, 2000), although its activity was lower than that of two other cationic antimicrobial polypeptides, lysozyme and defensin. Studies have also found that combinations of lysozyme-lactoferrin, lysozyme-SLPI and lactoferrin-SLPI were synergistic in their antimicrobial activity. The triple combination showed even greater synergy. Other combinations involving the human beta-defensins also display additive effects (Singh *et al.*, 2000).

Salivary SLPI is also a potent HIV-1 inhibitor that inhibits HIV-1 infection in macrophages. However, SLPI does not appear to bind HIV-1 viral particles, inhibit HIV-1

reverse transcriptase or protease, or transduce an activation signal or downregulate expression of the HIV-1 receptor, CD4 (McNeely *et al.*, 1995).

Investigations have revealed that macrophages and neutrophils are rich sources of SLPI (Jin *et al.*, 1998) and that its expression is induced in primary macrophages by LPS and suppressed by IFN- γ and that SLPI antagonises LPS-induced signalling and secretion (Jin *et al.*, 1997). Jin and colleagues (1998) have recently searched for regulators of SLPI expression, induced by or similar to LPS. They showed induction of SLPI by IL-10, IL-6 and LTA, and that the kinetics of its induction (slow to rise then prolonged) are consistent with the hypothesis that SLPI may act in an autocrine fashion as a brake on the response of macrophages to microbial inflammation.

LPS and LTA are bacterial cell wall constituents known for their cytokine stimulating activities in a number of mammalian cells (see sections 1.6.2.1 and 1.6.2.4). Both bacterial wall constituents bind CD14 (discussed in section 1.6.3.1). SLPI expression has been shown to inhibit both LPS- and LTA-induced nitric oxide production (Jin *et al.*, 1997 and Jin *et al.*, 1998) from macrophages suggesting that SLPI may bind CD14 in a manner that interferes with the binding of both LPS and LTA or their subsequent interactions with co-receptors (Jin *et al.*, 1998).

The human α -defensin HNP-1 (discussed later in section 1.7.2.3) significantly increases SLPI protein release by PBMCs in a time and dose-dependent fashion without affecting SLPI mRNA synthesis (VanWetering *et al.*, 1997).

1.7.2 Antimicrobial peptides

Another aspect of the innate immunity of vertebrates is the production of broad-spectrum antimicrobial peptides thought to be key effector molecules, which are particularly important in early defence against invading microorganisms (Boman, 1995, 1996).

Interest over many years has focused on the adaptive immune responses as being the main antimicrobial defence system. However, with its network of humoral and cellular responses, the adaptive immune system is found only in higher animals whereas innate immunity is encountered in all living beings (Hoffmann *et al.*, 1999). The discovery of mammalian antimicrobial peptides in the early 1980's led to a turning point in the appreciation of the innate immune system. Membrane-active proteins and peptides are now thought to play a crucial role in both the innate and adaptive immune system as antimicrobial agents.

Antimicrobial peptides are an ideal first line of defence since peptides are made more rapidly than proteins. Antimicrobial peptides may be made more than 100x faster than IgM, assuming a constant rate of peptide bond formation and since they are small peptides they also diffuse more rapidly (Boman, 1995 1996). These peptides, which are expressed in phagocytes and mucosal epithelial cells, are nearly all cationic and very often amphiphilic and kill their target by permeabilizing the cell membrane (Papo and Shai, 2003, Papagianni, 2003).

1.7.2.1 History of antimicrobial peptides

In 1939, Dubos demonstrated that 'an unidentified soil bacillus' produced some antibacterial product that could prevent pneumococcal infections in mice. In the following year Hotchkiss and Dubos reported the partial purification of the bactericidal substances produced by this soil bacterium, which was later identified as *Bacillus brevis*. In 1941-42 they described how, by starting from a *B. brevis* culture, they had purified and crystallised tyrocidine and gramicidin and shown them to be composed of amino acids, some of them D-amino acids. Gramicidin was also a forerunner to cecropins, defensins and magainins because it was the first peptide antibiotic found to induce the formation of voltage-gated anion channels in artificial membranes (Kriss and Biriuzova, 1951).

Cecropins and the defensins were the first antimicrobial peptides from animals to be characterized structurally. The cecropins were found in 1981 in the pupae of the *Cecropia* (a giant silk moth) and two years later defensins were identified in rabbit phagocytes (Steiner *et al.*, 1981, Selsted *et al.*, 1983). Subsequently antimicrobial peptides were identified in rat, human and guinea pig neutrophils and found to all belong to the α -defensin sub-class (Selsted *et al.*, 1984, Risso, 2000).

1.7.2.2 Classification of antimicrobial peptides

Gene-encoded, ribosomally-synthesised antimicrobial peptides are widely distributed in nature, being produced by mammals, birds, amphibia, insects, plants and microorganisms (Nissen-Meyer and Nes, 1997). They form a diverse group of peptides, as determined by their primary structures and, as mentioned previously, they are nearly all cationic and very often amphiphilic and kill their target by permeabilizing the cell membrane. It has been suggested that their positive charge facilitates interactions with the negatively charged bacterial phospholipid-containing membranes and/or acidic bacterial cell wall, whereas their amphiphilic character enables membrane permeabilization (Nissen-Meyer and Nes, 1997).

Many of these peptides may roughly be placed into one of three groups: 1) those that have a high content of one (or two) amino acid(s), often proline 2) those that contain intramolecular disulphide bonds, often stabilizing a predominantly β -sheet structure, and 3) those with amphiphilic regions if they assume an α -helical structure.

1.7.2.3 Defensins

The defensin family of antimicrobial peptides is an evolutionarily conserved group. In mammals, defensins include the α -defensins of the intestinal epithelium (HD-5 and HD-6)

and neutrophils (HNP-1 to HNP-4) and β -defensins of the skin and mucosal epithelia (h β D-1 to h β D-4) (Raj and Dentino 2002, Weinberg *et al.*, 1998, Ganz and Lehrer 1998).

The defensins are small cationic peptides, ranging in length from 29-42 amino acids. The β -defensins are 38-42 residues long and somewhat larger than the α -defensins which have 29-35 residues (White *et al.*, 1995). The α - and β -defensins differ in their number of residues, the location and connectivity of their cysteine residues and their unique consensus sequences. The α -defensins have been found in azurophilic granules of neutrophils and of some macrophages in humans, rabbits, rats and guinea pigs and may account for more than 5% of the total cellular protein (Lehrer *et al.*, 1993 and Selsted and Oulette 1995). These granule-associated defensins presumably kill phagocytosed microorganisms when the defensin-containing granules fuse with the phagocytic vacuoles containing the ingested microorganisms (White *et al.*, 1995).

Tracheal antimicrobial peptide (TAP) was the first member of the epithelial β -defensin family to be isolated and characterised (Diamond *et al.*, 1991). Originating in bovine tracheal mucosa, TAP is a 38-amino acid peptide, which when assayed *in vitro*, is bactericidal against Gram-negative and Gram-positive bacteria and also exhibits potent activity against *C. albicans* (Diamond *et al.*, 1991). Up-regulation of TAP mRNA was shown to occur via the CD14-mediated signal transduction pathway in bovine airway epithelial cells challenged with LPS, TNF- α or IL- β (Bevins *et al.*, 1996, Diamond *et al.*, 1996, Russell *et al.*, 1996). There is an NF- κ B site in the regulatory region of the TAP gene (Diamond *et al.*, 1991).

1.8 Human beta-defensins

Four human-beta defensins have been described. The first human-beta defensin 1 (h β D-1) was isolated from plasma, and has since been found in the pancreas, kidney, airway epithelium, salivary glands, prostate, placenta and endocervix of the female reproductive

tract. (Bensch *et al.*, 1995, Valore *et al.*, 1998). h β D-2 was originally isolated from psoriatic scale keratinocytes, basal h β D-2 mRNA expression has been detected in freshly isolated foreskin, lung and trachea (Harder *et al.*, 1997). This expression profile is nearly identical to that described for the tissue distribution of bovine TAP mRNA and in contrast to that seen in h β D-1.

Recently identified are h β D-3 and h β D-4 (Jia *et al.*, 2001 Garcia *et al.*, 2001a). h β D-3 has been identified in adult heart, skeletal muscle, placenta, skin, oesophagus, gingival keratinocytes, trachea and foetal thymus (Jia *et al.*, 2001), whilst h β D-4 shows tissue-specific, restricted expression in the testis, uterus, thyroid gland, lung and kidney during infection (Garcia *et al.*, 2001).

1.8.1 Antimicrobial action

Defensins exhibit antimicrobial, antifungal and antiviral activity. Analysis of synthetic h β D-1 showed it has broad-spectrum antimicrobial activity against Gram-negative organisms (*E. coli* and *Pseudomonas aeruginosa*) at concentrations similar to those described for α -defensins ie. 10-100 μ g/ml (Ganz and Lehrer 1994). The activity of h β D-1 is easily inhibited by salt and diminishes in the presence of >40mM NaCl (Goldman *et al.*, 1997). This salt-related inhibition is reduced at high concentrations of the protein (Singh *et al.*, 1998). h β D-1 is also active against some Gram-positive bacteria and *C. albicans* (Bensch 1995, Valore 1998). h β D-2 is functionally more targeted than h β D-1, being active against Gram-negative bacteria and the yeast *C. albicans*, but only bacteriostatic against the Gram-positive *S. aureus* (Harder *et al.*, 1997). h β D-2 is also approximately 10 times more potent than h β D-1 against *E. coli* (Singh *et al.*, 1998).

h β D-3, in contrast, has been shown to be active against Gram-negative and Gram-positive bacteria and *C. albicans* and is not significantly affected by increased NaCl concentrations

(Harder *et al.*, 2001). h β D-3 demonstrated potent antimicrobial activity against potentially pathogenic microorganisms including multi-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium*. (Harder *et al.*, 2001).

Synthetic h β D-4 exhibits a selective, NaCl-sensitive spectrum of antimicrobial activity, and it represents one of the most active antimicrobial peptides against *P. aeruginosa* (minimal inhibitory concentration: 4.1 μ g/ml) known to date (Garcia *et al.*, 2001a). h β D-4 inhibited the growth of Gram-positive *Staphylococcus carnosus*, Gram-negative *E. coli* and the yeast *Saccharomces cerevisiae*. Synergism between h β D-4 and h β D-3 has also been established. When assayed in conjunction with h β D-3, h β D-4 was found to have a additive antimicrobial effect against *S. carnosus* (Garcia *et al.*, 2001a).

The sensitivity of oral bacteria to killing by the human-beta defensins has yet to be established; however studies have investigated the effect of the human neutrophil peptides and protegrins. Strains of both *A. actinomycetemcomitans* and *Capnocytophaga* spp. have been found to be sensitive to synthetic protegrin (Miyasaki *et al.*, 1997). Other studies tested *F. nucleatum*, *P. gingivalis* and *P. intermedia*, all of which were found to be sensitive with E₉₉ (dose at which 99% killing occurs after 1h at 37⁰C) ranging from 0.5-19 μ g/ml. (Miyasaki *et al.*, 1998).

The human neutrophil peptides HNP-1 and HNP-2 were found to kill *Capnocytophaga* spp., whereas *A. actinomycetemcomitans* and *E. corrodens* strains were resistant (Miyasaki *et al.*, 1990). The human neutrophil peptides are less potent and more selective against periodontal pathogens than rabbit defensin NP-1. Killing of oral pathogens by the human defensins is slower and requires a greater concentration of peptide.

A peptide whose structure mimics the three-dimensional structure of large defensins has demonstrated microbicidal activity against oral microbes. A synthetic form of the dodecapeptide from bovine neutrophils displays antimicrobial activity at a concentration of

2-2.5mM against *P. gingivalis*, *A. actinomycetemcomitans*, *S. gordonii* and *S. mutans* (Raj *et al.*, 2000).

1.8.1.1 Mechanism of action of cationic antimicrobial peptides

Unlike conventional antibiotics, which destroy bacteria through various biochemical processes, cationic peptides work at a physical level, literally creating holes in the bacterial cell membrane to kill the bacterium (Nissen-Meyer and Nes, 1997, Papagianni, 2003, Papo and Shai, 2003). This physical attack destroys bacteria quickly, non-specifically and efficiently (figure 1.5). For years this led to the idea that it would, therefore, be extremely difficult for bacteria to develop resistance. However, reports published in only the last few years have shown that some bacteria are resistant. *S. aureus* resists human defensins by production of staphylokinase, which displays the ability to induce secretion of defensins, to bind them and to neutralize their bactericidal effect (Jin *et al.*, 2004). The oral bacterium *T. denticola* has also been shown to be resistant to killing by h β D-1 and h β D-2 (Brissette and Lukehart, 2002).

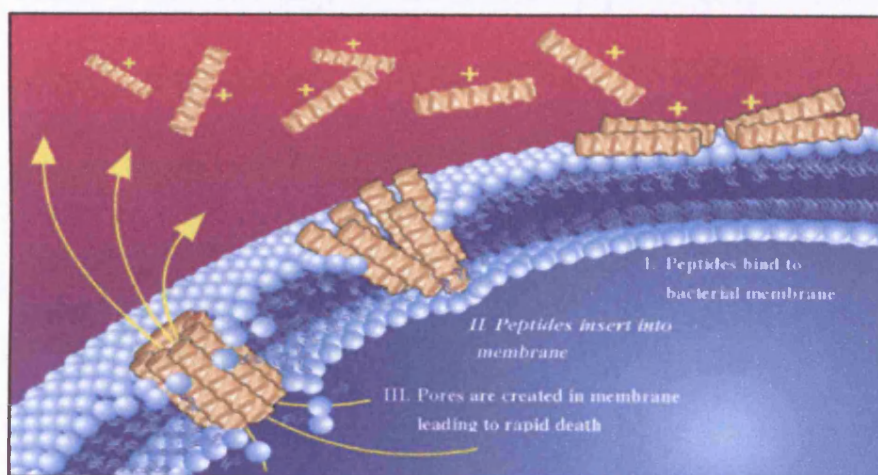


Figure 1.5 Mechanism of action of cationic antimicrobial peptide on membranes

From: http://www.mbiotech.com/tech_peptides.html

The antimicrobial action of human beta-defensins is dependent on their cationic charge and amphipathic structure with its polar (cationic) and hydrophobic surfaces. There is now circumstantial evidence that permeabilization of membranes is involved, either through the formation of multimeric pores as described for the α -defensins or by an electrostatic charge-based mechanism as suggested by the structural and electrostatic properties of h β D-2 oligomer (Harder *et al.*, 2001, Hoover *et al.*, 2000). They are thought to bind to bacterial outer membranes, integrate into the membrane, and then aggregate to form pores that leads to the death of the bacteria. Defensins and other antimicrobial peptides act synergistically with each other and with other types of antimicrobial agents, such as lysozyme and other antibiotics. In view of the increasing problem of bacterial resistance to traditional antimicrobials, this makes antimicrobial peptides particularly attractive as therapeutic agents.

The addition of two basic amino acids at the C-terminus and two hydrophobic amino acids, such as valine, at the N-terminus to an α -defensin sequence resulted in peptides that are optimally active against oral pathogens (Raj *et al.*, 2000). Sequence variation is currently being explored to develop therapeutic antimicrobial peptides for the treatment of mucositis (Chen *et al.*, 2000).

1.8.2 Expression and regulation of beta-defensins

The endogenous gene encoding the mammalian defensin TAP has been shown to characterise the response of tracheal epithelial cells (TECs) to bacterial LPS (Diamond *et al.*, 1996). It was also found that TECs express CD14 and that CD14 of epithelial cell origin mediates the LPS induction of TAP (Diamond *et al.*, 1996). h β D-2 was the first human beta-defensin found to be regulated at the transcriptional level in response to stimulation with TNF- α and specific microorganisms (Harder *et al.*, 1997). Studies are needed to understand the antimicrobial activity of h β D-1 and h β D-2 peptides against

relevant microorganisms and how the activity and expression may be altered in states such as periodontal diseases.

A previous study has determined whether the reduced microbial activity of neutrophils in patients with early-onset periodontitis is associated with a deficiency of nonoxidative microbicidal proteins (Flemmig and Miyasaki, 1994). It was shown that the human neutrophil peptide 3 (HNP-3) was significantly reduced in neutrophils from patients with generalised early-onset periodontitis. Due to the high variability of HNP1-3 in neutrophils of control subjects, the reduced HNP-3 content in neutrophils probably plays a minor role in the pathogenesis of generalised early-onset periodontitis (Flemmig and Miyasaki, 1994).

1.9 Aims of this thesis

The role of human beta-defensins in the oral cavity has only recently begun to be elucidated. It seems that their functions are more diverse than originally believed. Hence, along with their potent antimicrobial activity, it has also been shown that they are positioned to defend a number of mucosal sites, they are induced by a number of bacterial factors and inflammatory mediators, also more recently their mitogenic activity and role in adaptive immunity have been determined (Yang *et al.*, 1999, Yang *et al.*, 2002, Niyonsaba *et al.*, 2004). The 5' flanking region of h β D-1 contains NF-IL-6 and gamma interferon consensus sites, suggesting that its expression may be regulated by inflammatory mediators (Valore *et al.*, 1996). Since certain pro-inflammatory cytokines are upregulated in the periodontal diseases it may be that their expression is linked to that of the human beta defensins. The study of the oral production and expression of defensins and cytokines may help us to understand more clearly the innate immune mechanisms of the oral cavity. The properties of human beta-defensins described in the literature suggest the importance of determining the presence of these novel host antibiotics in gingival tissue, studying their

modes of regulation and their role in health and disease. It may be possible that the loss of function of the human beta-defensins may be a risk factor for periodontal diseases.

The determination of the expression profile of the human beta-defensins may prove important in determining whether the normal expression of these peptides can be enhanced. Characterisation of β -defensin regulation is essential for understanding the role of these peptides in protecting the host by activating both innate and adaptive immune systems and in contributing to the epithelial barrier in inflammatory disease processes.

The aims of this thesis were therefore:

- To determine the mRNA expression of healthy gingival tissue, and gingival tissue from patients with early-onset and chronic periodontal diseases, in particular cytokine and human beta-defensin expression.
- To localise the human-beta defensins in gingival sections from healthy patients and in those with periodontal diseases.
- To establish the role of viable and non-viable oral commensal bacteria and periodontopathogens in host innate immune protection by oral epithelial cells.
- To investigate the effect of various bacterial factors from oral commensal bacteria and periodontopathogens on the production of beta-defensins and IL-8 from oral epithelial cells, and the possible regulatory pathway involved.
- To determine the effect of bacterial adhesion and invasion on the production of human beta-defensins by oral epithelial cells.

Chapter 2

Materials and Methods

Chapter 2

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2.1 Cultivation of oral bacteria

All bacterial strains were maintained frozen at -70°C in trypticase soy broth (TSB) (Becton Dickinson) supplemented with 0.6% yeast extract (YE) (Oxoid, Basingstoke, UK) and 10% glycerol (BDH). After approximately every four weeks fresh stocks were grown from frozen supplies. Cultures were checked weekly both visually and by Gram-staining for contamination with other bacteria.

2.1.1 *Streptococcus gordonii*

Strains used in this study were *Streptococcus gordonii* DL1 (Challis) wild type and mutants of *Streptococcus gordonii* DL1 (Challis) in which the genes encoding high-molecular-mass cell surface polypeptides CshA and CshB or SspA and SspB had been inactivated (CshAB- and SspAB-), see table 1 (McNab *et al.*, 1994, Demuth *et al.*, 1996). Additionally, a *S. gordonii* quad-mutant was also used, in which genes for CshA, CshB, SspA and SspB had been inactivated. Further, a *S. gordonii* quint-mutant was used which, in addition to the aforementioned proteins, also has a fibronectin binding protein (FbpA) knocked out (Christie *et al.*, 2002). All strains were provided by Dr Rod McNab of the Eastman Dental Institute, University College London, UK and details are given in Table 1. Strains were maintained by twice-weekly subculture on TSB-YE agar plates with 10% defibrinated horse blood (TCS Biosciences, UK) and cultivated at 37°C in a humidified atmosphere which contained 5% CO_2 in air. For the purpose of the experiments, all *S. gordonii* strains were grown in Todd-Hewitt broth (Oxoid, Basingstoke, UK) with incubation at 37°C in an atmosphere which consisted of 5% CO_2 in air.

Strain	Genes knocked out	Code	Resistance	Comments
<i>S. gordonii</i> DL1 Challis	Wild type (none)			
<i>S. gordonii</i> DL1 Challis	CshA CshB	OB277	Erythromycin 1µg/ml Chloramphenicol 5µg/ml	McNab <i>et al.</i> , 1994
<i>S. gordonii</i> DL1 Challis	SspA SspB	OB219	Erythromycin 1µg/ml	Demuth <i>et al.</i> , 1996
<i>S. gordonii</i> DL1 Challis (quad)	CshA CshB SspA SspB	OB392	Erythromycin 1µg/ml Chloramphenicol 5µg/ml Kanamycin 200µg/ml	Christie <i>et al.</i> , 2002
<i>S. gordonii</i> DL1 Challis (quint)	CshA CshB SspA SspB FbpA	CB512.1	Spectinomycin 100µg/ml	<i>S. gordonii</i> OB392 transformed with chromosomal DNA from UB1245 Christie <i>et al.</i> , 2002

Table 1 Strains of *Streptococcus gordonii* DL1 Challis used in this study

2.1.2 *Streptococcus sanguis*

Streptococcus sanguis NCTC 10904 used in this study was provided by Dr. Rod McNab of the Eastman Dental Institute, University College London, UK. The strain was maintained by twice weekly subculture on TSB-YE agar plates supplemented with 10% defibrinated horse blood and cultivated at 37°C in an atmosphere consisting of 5% CO₂ in air. For the purpose of the experiments, *S. sanguis* was grown in Todd-Hewitt broth.

2.1.3 *Actinobacillus actinomycetemcomitans*

SUNY 465, the *A. actinomycetemcomitans* invasion prototype and SUNY 462, the *A. actinomycetemcomitans* non-invasive prototype originated from the laboratory of J.J Zambon, State University of New York, Buffalo, USA and were provided by Dr. P. Fives-Taylor, University of Vermont, USA. For the purpose of the experiments, the *A. actinomycetemcomitans* strains were grown in TSB-YE broth. Solid medium was prepared by adding technical agar N° 3 (Oxoid, Basingstoke, UK) to a final concentration of 1.5% (wt/vol) and 10% defibrinated horse blood. Strains were maintained by twice

weekly subculture on TSB-YE agar and cultivated at 37°C in an atmosphere consisting of 5% CO₂ in air.

2.1.4 *Porphyromonas gingivalis*

P. gingivalis W50 was kindly supplied by Miss Marilou Ciantar, Eastman Dental Institute, and was maintained by weekly subculture on Wilkins-Chalgren agar (Oxoid Ltd., Basingstoke, UK) plates supplemented with 10% defibrinated horse blood (Oxoid Ltd., Basingstoke, UK), and incubated anaerobically at 37°C. (All anaerobic subcultures and broth cultures were stored in a compact anaerobic work station, Don Whitley Scientific Ltd., West Yorkshire, UK).

2.2 Human cell culture

2.2.1 Culture of oral epithelial cell line H357

Oral epithelial cell line H357 was established from a tongue squamous cell carcinoma (Sugiyama *et al.*, 1993). The cell line originated from the laboratory of S.S Prime, University of Bristol Dental School, Bristol, UK and was provided by Miss Josie Furness, Dept. of Oral Pathology, The Eastman Dental Institute. H357 cells grown in 75-cm² tissue culture flasks (Nunc, Sarstedt, UK) viewed at x100 magnification can be seen in Figure 2.1. Cells were maintained in keratinocyte growth medium (KGM) which consisted of 1 part Hams's F12 medium plus 3 parts of Dulbecco's modified Eagle's medium (Gibco, UK), supplemented with 1.8x10⁻⁴M adenine (Sigma, UK), 10% foetal calf serum (FCS, PAA Laboratories, Yeovil, UK), 100IU penicillin, 100µg/ml streptomycin (Gibco, UK), 2.5µg/ml fungizone (Gibco, UK), 0.5µg/ml hydrocortisone (Sigma, UK), 5µg/ml insulin (Sigma, UK), 10⁻¹⁰M cholera toxin (Sigma, UK) and 10ng/ml epidermal growth factor (Sigma, UK). Cells were cultured in 75-cm² flasks at 37°C in a humidified atmosphere of 5% CO₂ in air. Twice weekly, cells were split at a ratio of 1:8 by treatment with trypsin-

EDTA (0.25%, Invitrogen, UK) to detach cells. The purity of the H357 cell line was not determined during the course of all experiments in this thesis.



Figure 2.1 Cell line H357 viewed under an inverted microscope at a magnification of x100

2.2.2 Preparation of peripheral blood mononuclear cells from buffy coat blood

Mononuclear cells were prepared by Ficoll-Hypaque of buffy coat blood residues (Ferrante and Thong, 1978). This method separates blood leukocytes according to their size and density. Monocytes were purified further by adherence.

Reagents

- RPMI 1640 (Invitrogen, UK)
- RPMI 1640 with 100IU/ml penicillin/streptomycin (Invitrogen, UK) 2mM L-glutamine (Invitrogen, UK) and 2% heat-inactivated foetal calf serum (HIFCS), (ICN, Thame, UK)
- Histopaque® 1077, containing polysucrose and sodium deatrizoate at a density of 1.077g/ml (Sigma, UK)
- Red cell lysis buffer (8.3g/L ammonium chloride in 0.01M Tris-HCl)

Procedure

An equal volume of warm RPMI 1640 (25ml) was carefully added to mixed buffy coat blood residues (obtained from The Blood Transfusion Centre, Colindale, London, UK) in sterile 50ml centrifuge tubes (Sarstedt), which were then inverted three times to mix. 35ml of blood/RPMI 1640 suspension was carefully layered onto 15ml Histopaque® in a sterile 50ml centrifuge tube. Tubes were centrifuged at 1500g for 30 minutes at room temperature. This step required the centrifuge deceleration to be deactivated in order to avoid sudden jolts that may disrupt the gradient.

The upper layer was removed and discarded. Next 10-12ml of the mononuclear leukocyte cell layer was carefully removed and placed into a 50ml centrifuge tube which was filled with RPMI 1640 and centrifuged at 1700g for 15 minutes, this time with deceleration in order to obtain a solid pellet.

The supernatant from each tube was removed and the pellets gently resuspended in 5ml of red cell lysis buffer and the tube incubated at room temperature for 10 minutes. After incubation, the tubes were filled with RPMI 1640 and centrifuged at 1700g for 15 minutes. The resulting pellets were pooled into one tube and then washed twice in RPMI 1640, as above. The pellet was then resuspended in RPMI 1640 containing 100U/ml penicillin/streptomycin, 2mM L-glutamine and 2% heat-inactivated foetal calf serum (HIFCS). Cells were then counted and cultured in tissue flasks or plates depending on the assay.

2.3 Detection of human IL-8 by ELISA

Reagents

All chemicals were purchased from BDH, UK unless otherwise stated

- **Wash/dilution buffer pH 7.2**

0.5M NaCl, 2.5mM NaH₂PO₄, 7.5mM Na₂HPO₄, 0.1%v/v Tween 20

- **Substrate buffer pH 5.0**

34.7mM citric acid, 66.7mM Na₂HPO₄

Antibodies

Antibodies were provided by Dr. Stephen Poole, National Institute for Biological Standards and Control, South Mims, Potters Bar, Hertfordshire, UK

- **Coating antibody:** Immunoaffinity-purified polyclonal antibodies from sheep (anti-human IL-8 serum S333/BM). For coating plates, a stock solution of coating antibody was diluted to 2µg/ml in phosphate buffered saline (PBS) (Gibco, UK).
- **Detecting (developing) antibody:** The same purified antiserum was used after biotinylation.

Procedure

Coating antibody was diluted with PBS and 100µl aliquots were added to each well of a 96-well plate (Immunoplate Maxisorp, NUNC). The plate was wrapped in foil and left at room temperature for 2-4 h or at 4°C for 16-24 h. Dilutions of samples and standards were prepared in wash buffer. Samples were diluted 1:10 unless otherwise stated. The standards used ranged from 0-10,000pg/ml. The plate was then washed 3 times by filling each well with 200µl wash buffer, inverting over a sink, and tapping dry with absorbent paper. 100µl of sample or standard was then added to each well. The plate was once again covered using foil and incubated at 37°C for 2h. The plate was washed 3 times with wash/dilution buffer as previously described. 100µl/well of biotinylated antibody diluted 1:2000 with wash/dilution buffer was added and incubated at 37°C for 1h. The plate was washed 3 times with wash/dilution buffer as previously described.

100µl of avidin-horseradish peroxidase (Avidin-HRP, Dako Ltd, UK) diluted 1:4000 with wash/dilution buffer was added to each well and incubated at 37°C for 15 minutes. The plate was washed 3 times with wash/dilution buffer, as previously described. 100µl of peroxidase substrate 1, 2-phenylenediamine dihydrochloride (OPD, Sigma, Poole, UK) (400µg/ml) dissolved in substrate buffer, containing 0.4µl/ml hydrogen peroxide (Sigma, Poole, UK) was added to plates. The plate was covered with foil and left in the dark for 15-20 minutes or until sufficient colour had developed. 100µl of 1M sulphuric acid was added to each well to stop the enzyme reaction. The optical density was measured at 490nm using a Dynex plate reader. A standard curve of IL-8 was generated using the Revelation software package (Dynex Technologies, Virginia, USA).

2.4 Preparation of total cellular RNA

Reagents

- **Solution D**

4M guanidinium thiocyanate (Sigma, UK)

25mM sodium citrate, pH 7.0 (BDH, UK)

0.5% sodium sarcosyl (BDH, UK)

0.75% β -mercaptoethanol (added just before use), (Sigma, UK)

in diethylpyrocarbonate(DEPC) treated water

Procedure

From tissue

Tissue samples were minced using sterile scalpel blades on a glass slide (cleaned with alcohol) with a drop of solution D and then placed into 1.5ml Eppendorfs containing 600 μ l of solution D and homogenised using a sterile Eppendorf pestle.

From cells

Solution D was added directly to cell monolayers in tissue culture plates. Cell lysates were scraped from each well and placed into sterile 1.5ml Eppendorfs.

RNA was extracted using a method adapted from Chomczynski and Sacchi, 1987. To the cell/tissue lysate the following were then added to each Eppendorf sequentially: 50 μ l of 3.0 M sodium acetate (pH 5.0), (Sigma, UK), 500 μ l of citrate-saturated phenol, (Sigma, UK) (containing 0.01% hydroxyquinoline (BDH, UK)), 100 μ l of 49:1 chloroform/isoamylalcohol (Sigma, UK). Samples were vortexed for 10 seconds to ensure complete mixing of all components and incubated on ice for 10 minutes. Samples were then centrifuged at 13,000rpm for 10 minutes; the upper aqueous phase (about 500 μ l) recovered and precipitated by addition of an equal amount of isopropanol (BDH, UK), with thorough mixing and incubation at -20°C for 1-2 hours. The precipitate was recovered by

centrifugation again at 13,000rpm for 30 minutes. The recovered pellet was dissolved by addition of 150µl of Solution D with thorough mixing and re-precipitated by addition of 150µl isopropanol. Samples were placed at -20°C for 1 hour. The precipitate was again recovered by centrifugation of the samples at 13,000rpm for 30 minutes. The final RNA pellet was washed with 70% ethanol (Sigma, UK) and used to generate cDNA.

2.5 Reverse transcription of mRNA

After removal of ethanol, samples were air dried at room temperature and then dissolved in 50µl of water and the RNA concentration measured spectrophotometrically. For reverse transcription, 5µg of RNA in 15µl of DEPC-treated water from each sample was used. 1µl of OligodT (Sigma Genosys, UK) was added and samples were heated to 70°C for 10 minutes to denature the RNA. The Eppendorfs containing reaction mixture were then placed on ice to chill and 7µl of master mix I, 4µl first strand buffer, 2µl dTT (Gibco BRL, UK), 1µl 10mM dNTPs (Gibco BRL, UK) were added. After addition of master mix I, samples were transferred to 42°C and incubated for 2 minutes. 5µl of master mix II; 0.5 µl Superscript II reverse transcriptase (Gibco BRL, UK) and 4.5µl DEPC-treated water was added and the samples incubated at 42°C for a further hour. Samples were heated to 70°C for 10-15 minutes to inactivate the reverse transcriptase, then diluted 1:4 with DEPC water and stored at -20°C.

2.6 Polymerase chain reaction

Transcripts for all genes were determined by PCR using 5µl of the cDNA template from samples prepared as above. 45µl of master mix containing 5µl PCR buffer (10x), 1.5µl MgCl₂ (50mM), 1µl dNTPs (dATP, dCTP, dGTP, dTTP each at a concentration of 25 mM), 0.5µl Taq polymerase (Gibco BRL, UK), 0.5µl forward primer (50µM), 0.5µl reverse primer (50µM), 36.5µl pyrogen free/DECP-treated water was added to each sample. Tubes were gently vortexed and placed into an automated DNA thermal cycler with a heated lid (Eppendorf Mastercycler). Programs and primer sequences are shown in Table 2, all primers were purchased from Sigma-Genosys, UK. PCR parameters were taken from the same publications as the primer sequences for each primer pair. The annealing temperature for TLR4 was determined using 50, 52, 54 and 56⁰C (based around the predicted T_m of the primers provided by Sigma-Genosys) in separate PCR reactions to find the optimum temperature. The assumption of a positive PCR product was based on the predicted size of the particular amplicon.

Table 2. *Primer sequences and PCR product size*

Target mRNA	Primers	Annealing	Extension	Size of PCR product (bp)	Reference
hβD-1	F 5' tctgagatggcctcaggtggtaac 3' R 5' ataactcaaaagcaatttcctttat 3'	60	72	253	Zhao <i>et al.</i> , 1996
hβD-2	F 5' ccagccatcagccatgagggt 3' R 5' ggagccctttctgaatccgca 3'	65	72	252	Harder <i>et al.</i> , 1997
GAPDH	F 5' ccacccatggcaaattccatggca 3' R 5' tctagacggcaggtgaggtccacc 3'	60	72	610	Provided by Dr. P. Tabona
IL-6	F 5' atgaactccttctccacaagcgc 3' R 5' gaagagccctcaggctggactg 3'	60	60	628	Jung <i>et al.</i> , 1995
IL-8	F 5' atgacttccaagctggccgtggct 3' R 5' tctcagccctcttcaaaaacttctc 3'	60	60	289	Jung <i>et al.</i> , 1995
IL-1β	F 5' aaacagatgaagtgtctcctccagg 3' R 5' tggagaacaccacttgttgctcca 3'	60	60	388	Jung <i>et al.</i> , 1995
IFN-γ	F 5' atatcttggtttcagc 3' R 5' ctctttttcgcttcctgt 3'	60	60	501	Jung <i>et al.</i> , 1995

Target mRNA	Primers	Annealing	Extension	Size of PCR product (bp)	Reference
IL-10	F 5' atgcccgaagctgagaaccaagaccca 3' R 5' tctcaaggggctgggtcagctatccca 3'	72	72	352	Jung <i>et al.</i> , 1995
TLR2	F 5' gccaaagtcttgattgattgg 3' R 5' ttgaagtctccagctcctg 3'	54	72	347	Zhang <i>et al.</i> , 1999
TLR4	F 5' tggcatgaaaccagagctttc 3' R 5' tgccccatcttcaattgtctgg 3'	54	72	291	Primers were designed from TLR4 mRNA Genbank accession number U88880
SLPI	F 5' ccttagatacaagaaacctga 3' R 5' ccatgcaacacttcaagtcacgc 3'	65	72	230	Finkbeiner <i>et al.</i> , 1993
CD14	F 5' ggtgccgctgtgtaggaaaga 3' R 5' ggtcctcgagcgtcagttcct 3'	63	72	450	Cario <i>et al.</i> , 2000

2.7 Agarose gel electrophoresis

2.7.1 Preparation of 1% agarose gel

Reagents

All chemicals were purchased from Sigma, UK unless otherwise stated

- **TBE buffer (5X)**

54g Tris base

27.5g boric acid

3.72g EDTA

1 litre distilled water

Into a flask, 1g agarose powder (Bioline, UK) and 100ml TBE buffer (1x) were placed and heated in the microwave for 2 minutes. Once dissolved, the gel was cooled to hand temperature and 10 μ l of ethidium bromide (500 μ g/ml (Sigma, UK) added. The gel was carefully poured into a gel casting unit, the appropriate sized comb was then placed into the unit and the gel allowed to set for 1 hour.

2.7.2 Visualisation of PCR products

To each sample, 12.5 μ l of sample buffer (bromophenol blue (Sigma, UK), 20% glycerol (BDH, UK), tris borate EDTA [TBE]) was added and then mixed. Once the gels had set, they were placed into a gel system (11/14 horizon gel system from Life Technologies) with 800ml of TBE buffer (1x). 20 μ l of PCR product was loaded into the wells and run for 60 minutes at 100 volts (50 mAmps). Gels were visualised and photographed under UV illumination, using an AlphaImager photo system (Alpha Innotech, Cannock, UK).

2.8 Collection and processing of gingival biopsy specimens

Samples of gingival tissue were collected from the Department of Periodontology at the Eastman Dental Institute. These were placed in 10ml of the tissue culture liquid, DMEM (Gibco, UK), directly after surgical removal. Within an hour, tissue samples were placed in 4% paraformaldehyde (BDH, UK) to fix the tissue. After fixation, the tissue was processed into a form in which it could be cut into sections by embedding the fixed tissue into paraffin wax.

2.8.1 Paraffin embedding

The tissue samples were placed into labelled plastic cassettes, which were then placed into an automatic tissue processor, the Shandon Citadel®.

The processing machine automatically transferred the cassettes in a basket through the following steps:

1. 70% alcohol – 1 hour
2. 90% alcohol – 1 hour
- 3-6. 100% alcohol - 1½ hours
- 7+8. Xylene - 1¾ hours
9. Xylene - 1½ hours
- 10+11. Molten wax - 2½ hours

Steps 1-6 give gentle but complete dehydration to remove aqueous fixative and any tissue water content. Steps 7-9 expose tissues to the 'clearing' fluid, which is totally miscible with the dehydrating alcohol and wax embedding agent. Steps 10 and 11 impregnate the tissue with molten wax for the final embedding stage which sets specimens in blocks of paraffin wax from which sections may be cut.

2.8.2 Immunostaining of paraffin-embedded gingival sections for the human β -defensins, h β D-1 and h β D-2

Reagents

All chemicals were from BDH unless otherwise stated

- **Tris-buffered saline**

For 1 litre

7.88g of NaCl in 900ml dH₂O

6.06g Trizma HCl and 1.39g Trizma base in 100ml dH₂O

Add together to make 1 litre TBS

- **Antibodies**

Rabbit anti-h β D-1 (5th boost for histochemical stain only)

Rabbit pre-immune serum for h β D-1

Rabbit anti-h β D-2

Goat anti-rabbit secondary antibody – HRP conjugated (Dako, UK)

The anti h β D-1 and pre-immune serum for h β D-1 were kindly provided by Professor Tomas Ganz and methods provided by Dr. Erika Valore, both at the Department of Medicine and Will Rogers Institute for Pulmonary Research, UCLA, USA. (for ref see Dale *et al.*, 2001).

The anti-h β D-2 was kindly provided by Dr. M. Nakazato, Miyazaki Medical College, Miyazaki, Japan (Hiratsuka *et al.*, 1998).

Procedure

All chemicals were purchased from BDH unless otherwise stated.

Sections were cut from paraffin-embedded tissues at 5µm using a sledge microtome (AS300, Shandon, UK) and floated on a 40°C water bath containing distilled water. The sections were transferred onto 3-amino propyl tri ethoxysilane (APES) coated slides (see procedure below) and placed onto a hot plate (60°C) for approximately 20 minutes (so the wax just started to melt) to bond the tissue to the glass.

APES treatment of slides

Glass slides were immersed in 1% acid/alcohol (1% (v/v) concentrated HCl, 70% ethanol, 29% H₂O) for 30 mins. The slides were first rinsed in running tap water, then immersed in distilled water, after which they were allowed to dry. Slides were then immersed in acetone for 10 minutes. Next, slides were immersed in 2% (v/v) APES in acetone for 5 minutes. Slides were then briefly rinsed in 2 sequential volumes of distilled H₂O, left to dry and stored ready for use.

Tissue sections were then deparaffinised by sequentially placing slides in:

xylene	2X 3min
100% ethanol	2X 3min
95% ethanol	1X 2min
70% ethanol	1X 2min
tap water	1X 2min
dH ₂ O	1X 2 min

The slides were then washed for 5 minutes in TBS and then treated to remove endogenous peroxidase by exposure to 1% hydrogen peroxide (30% v/v, Sigma, UK) for 5 minutes and then transferred directly to 0.02% aqueous sodium borohydride, incubated for exactly 2

min and rinsed with TBS. Slides were incubated in the first antibody solution (see below for details) overnight at room temperature in a humidified environment.

Antibody solution

1% gelatin (type B: bovine skin bloom, Sigma, Poole, UK) was melted in TBS by gentle heating and 0.05% Tween 20 and 0.01% thimersol added. Antibody dilution for the human- β defensins was 1:1000 and pre-immune serum was used as a negative control, also at a dilution of 1:1000.

Slides were washed in TBS/0.05% Tween 20, for 20 minutes, 3 times, with gentle agitation and again incubated overnight with the secondary antibody (HRP conjugated goat-anti rabbit) diluted 1:2000 in antibody solution as described above. Slides were washed in TBS/0.05% Tween 20, for 20 minutes, 3 times, with gentle agitation and then rinsed in dH₂O. Slides were developed in fresh diaminobenzidine (DAB) solution for 1-2 mins. 30mg of DAB was dissolved in 50ml 50mM Tris pH 7.6 and 50 μ l 30% hydrogen peroxide added. The DAB solution was added immediately to slides and development stopped by rinsing in water. Slides were checked for the desired level of staining prior to counterstaining.

Counterstain

Incubate slides in Harris haematoxylin stain	20-30 sec
Wash with running tap water	1 min
0.05% conc. HCl in 70% alcohol	agitate a few seconds
Wash with running tap water	10 min
Dehydrate and mount	

2.9 Flow Cytometry

Flow cytometry (outlined in Figure 2.2) measures the physical properties of cells and their fluorescence characteristics. The size and the internal complexity of cells provide detectable light scatter signals. Fluorescence can be introduced by the binding of dyes to cellular components or the binding of antibody-conjugated fluorescent dyes to the proteins on the cell surface or inside the cell. Fluorescent-labelled cells are then carried in isotonic fluid through a light source, e.g a laser beam. The fluorochromes are excited to a higher energy state to emit light at higher wavelengths. Emitted light signals are collected via optics and directed through a series of filters and dichroic mirrors to isolate signals with the wavelength of interest. Finally the signals are detected by photomultipliers and digitised for computer analysis.

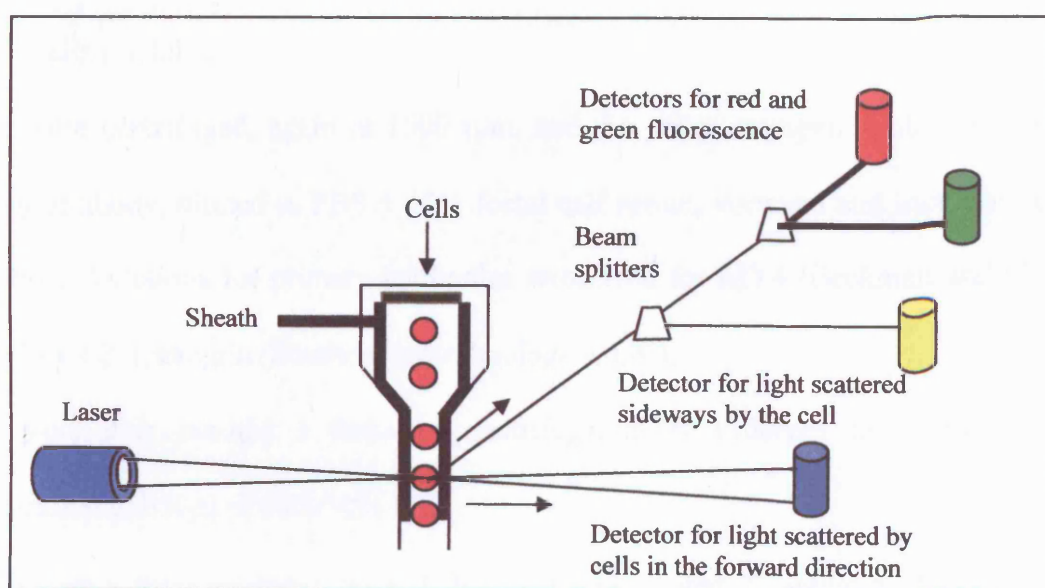


Figure 2.2 Schematic representation of a flow cytometer

2.9.1 Analysis of H357 and PBMC cells for surface CD14 and β -1 integrin

2.9.1.1 Cell preparation and labelling

Reagents

- FITC conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins (Dako UK)
- β -1 integrin antibody, mouse anti-human CD29 (Southern Biotechnologies, UK)
- MY4 (IgG2b mouse anti-human CD14, Beckman and Coulter Immunology, UK)

Procedure

Confluent H357 monolayers were trypsinised from T75 flasks with 1X trypsin-EDTA (0.25%, Invitrogen, UK), washed from the flask with fresh KGM and placed into a 15ml tube. Cells were centrifuged at 1500 rpm, resuspended in PBS, counted and placed into FACS tubes (Falcon, BDH, UK) at a density of 500,000 cells per tube. PBMCs were prepared as described in section 2.2.2 and also placed into FACS tubes but at a density of 1×10^6 cells per tube.

Tubes were centrifuged, again at 1500 rpm, and the pellet resuspended in 250 μ l of the primary antibody, diluted in PBS + 10% foetal calf serum, vortexed and incubated on ice for 1 hour. Dilutions for primary antibodies were 1:40 for MY4 (Beckman and Coulter) and 1:25 for β -1 integrin (Southern biotechnologies, UK).

Cells were then washed 3 times by centrifugation at 1500rpm for 3 minutes and resuspension in 250 μ l of PBS/10% FCS.

Samples were then incubated with 250 μ l FITC-conjugated 2^o antibody diluted 1:50 in PBS/10% FCS. After addition of antibody, samples were then vortexed and incubated on ice for 30 minutes.

Cells were again washed three times by centrifugation at 1500rpm for 3 minutes and resuspension in 250 μ l PBS/10% FCS.

Cells were resuspended in 500µl of PBS/10% FCS and subjected to pipetting to ensure a single cell suspension and then analysed using the FACScan cytometer (Becton Dickinson).

2.9.1.2 Data acquisition and analysis

Acquisition

The FACScan cytometer (Becton Dickinson, Oxford, UK) was set up according to the manufacturer's recommendations and used in conjunction with the CellQuest data acquisition software. The graphs below (Figure 2.3) demonstrate a typical representation of fluorescence detection and a density plot of both oral epithelial cells and monocytes which show how results will be represented. Results were further analysed using WinMDI software.

Data analysis

Initially a density plot of cellular size against granularity was produced for control cells. From a density plot, a population was determined corresponding to single cells excluding cell clumps and debris (Figure 2.3 left graph). The area of this plot corresponding to this population was gated (labelled R1 on both graphs) and subsequent analysis referred only to events falling within this gated area, see Figure 2.3 for oral epithelial cells. A typical monocyte population can be seen also in Figure 2.3, monocytes were also identified and gated according to their forward and light scattering properties. Monocytes are larger and more granular than T-cells and hence have higher levels of forward and side scatter. (Hoffman *et al.*, 1980, Peakman *et al.*, 1993).

For each test, a histogram of the gated population was drawn displaying FITC fluorescence (a measure of the amount of antigen detected per cell) against events (the number of cells counted) and compared against the negative isotype control, an example can be seen in

Figure 2.3, see bottom graph. Statistical analysis was performed by WinMDI and data displayed under the corresponding histogram plot. Results show the percentage of fluorescent-positive cells compared to the control isotype.

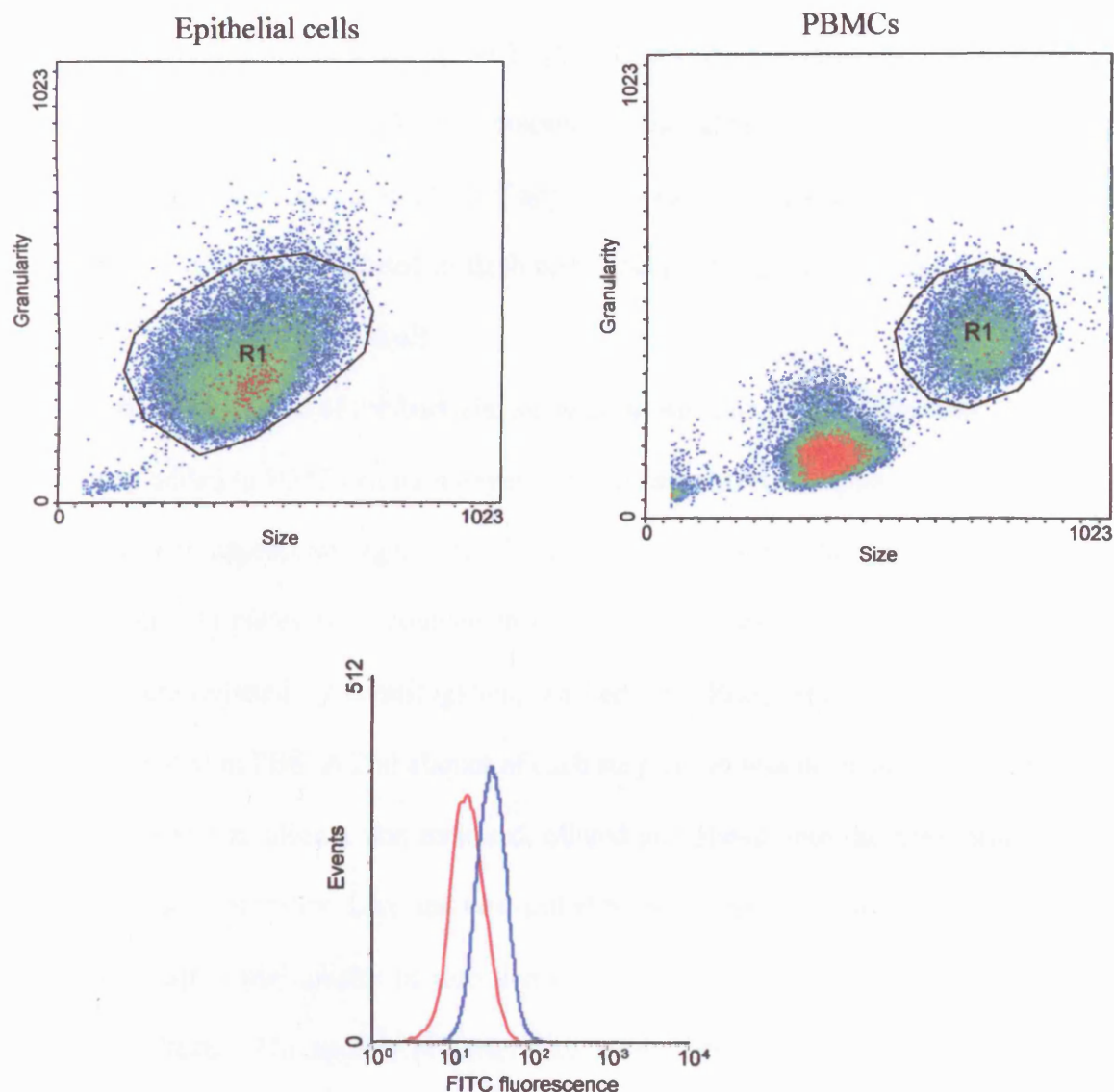


Figure 2.3 Representative FACS analysis plots

Typical dot plots (top) of PBMC with a monocyte population gated ((R1) right) and an oral epithelial cell line gated ((R1) left), and a typical histogram (bottom). All were created using WinMDI software to analyse results obtained with the FACSscan cytometer used in conjunction with CellQuest data acquisition. (Top) Oral epithelial cells and monocytes are gated (ie. an area corresponding to the size and granularity of a homogenous cell population is defined; R1). (bottom) Fluorescent values of cells within the gated regions are determined by statistical analysis using WinMDI software, the graph shows peaks for the isotype control (red) and the number of fluorescent positive cells (blue).

2.10 Incubation of oral epithelial cells with live and heat-killed bacteria

Approximately 1.25×10^5 H357 cells in KGM were seeded into 6-well tissue culture plates (Nunc, Sarstedt, UK) and incubated for 40h until confluent. Bacterial strains, except *P. gingivalis*, were inoculated into 10ml of the appropriate broth and grown in specific conditions; see section 2.1 for growth conditions.

After 6 days growth anaerobically on FAA plates, a few colonies of *P. gingivalis* W50 were inoculated into sterile PBS. The suspension was adjusted to an OD of 0.2 at 560nm, which corresponds to a concentration of approximately 10^8 bacteria/ml. Overnight cultures of all other bacteria were diluted in fresh broth and grown to exponential growth stage, as determined spectrophotometrically.

At this point, an aliquot of the bacterial suspension was removed to determine the number of bacteria added to H357 cell monolayers retrospectively. The aliquot was serially diluted and plated onto appropriate agar. After 5 days incubation under the appropriate conditions (see section 2.1) plates were counted to determine the CFU/ml used in the experiment. Bacteria were pelleted by centrifugation, washed with PBS, repelleted by centrifugation and resuspended in PBS. A 2ml aliquot of each suspension was incubated at 60°C for 1h to kill the cells and an aliquot was removed, diluted and plated onto the appropriate agar to determine loss of viability. Live and heat-killed bacteria were then added to antibiotic-free KGM to obtain a multiplicity of infection of 1, 10 and 100 bacteria to 1 epithelial cell (each in triplicate). The number of bacteria added to H357 monolayers was judged on the OD of bacterial cultures and previously determined CFU/ml at a particular OD. Epithelial cell numbers for each experiment were determined by trypsinising a confluent H357 monolayer from the tissue culture plate and the cells counted using a haemocytometer. Bacteria were centrifuged onto the monolayer at 2000rpm for 10 minutes at room temperature and then incubated at 37°C in an atmosphere containing 5% CO_2 for 5h.

2.11 Invasion/adherence assay

Approximately 1×10^5 H357 cells in KGM were seeded in 24-well tissue culture plates (Nunc, Sarstedt, UK) and incubated for approximately 24h until a confluent monolayer could be seen. Bacteria from an overnight culture were diluted in fresh broth and harvested during the early exponential growth phase, as determined spectrophotometrically. At this point, an aliquot of the culture was removed, serially diluted and plated onto appropriate agar. After 5 days incubation at 37°C in an atmosphere containing 5% CO_2 , plates were counted to determine the CFU/ml. Bacteria were pelleted by centrifugation, washed with PBS, repelleted by centrifugation and suspended in antibiotic-free KGM. Cell monolayers were washed with PBS and inoculated with bacterial suspensions that had been adjusted to obtain multiplicity of infection of 1, 10 and 50 bacteria to 1 epithelial cell (each in triplicate). Bacteria were centrifuged onto the monolayer at 2000rpm for 10 minutes at room temperature and then incubated at 37°C in an atmosphere containing 5% CO_2 for 2h. To determine the number of cell-associated bacteria, cell monolayers were washed 6 times with PBS to remove all unattached extracellular bacteria and 0.1ml of 0.5% Triton X-100 (BDH) in PBS was added to lyse cells. After lysis, 2.0ml of PBS was added to each well to dilute the detergent, appropriate dilutions were made and spread on TSB-YE plates with 10% horse blood in duplicate, and after 5 days incubation at 37°C in an atmosphere containing 5% CO_2 , CFU were enumerated.

To determine intracellular bacteria, cell monolayers were washed 3 times with PBS and incubated in the presence of cell culture medium containing $200\mu\text{g/ml}$ gentamycin (to kill all extracellular bacteria) for 1h at 37°C in an atmosphere containing 5% CO_2 . Cell monolayers were then washed 3 times with PBS and processed as for total cell-associated bacteria.

Chapter 3

Antibiotic peptide and cytokine levels in human periodontal diseases

Chapter 3

Antibiotic peptide and cytokine levels in human periodontal diseases

3.1 Introduction

The current understanding of the pathogenesis of periodontal disease suggests that tissue may be destroyed following the modulation of host defences by bacterial and host products which stimulate the host inflammatory process (Dennison and Van Dyke 1997). The local host response to oral bacteria includes the recruitment of leukocytes and the subsequent release of inflammatory mediators and cytokines, which appear to play crucial roles in the pathogenesis of periodontal diseases. Cytokines, which play important roles in inflammatory responses, are also prominent regulators of normal tissue homeostasis. Messenger RNA expression of cytokines that are associated with periodontitis has been detected in clinically healthy gingival tissues (Okada *et al.*, 1996).

Several studies have demonstrated that IL-1, IL-6, IL-8 and TNF- α levels are increased in the periodontal tissue and/or gingival crevicular fluid from diseased sites compared to those in healthy sites (Masada *et al.*, 1990, Stashenko *et al.*, 1991 Tsai *et al.*, 1995). It is also reported that the total quantity of these cytokines is markedly reduced following effective periodontal treatments. Lundqvist and co-workers (1994) showed that gingival epithelial cells freshly isolated from normal and inflamed gingiva expressed similar levels of IL-1 β , IL-6, IL-8, TNF- α and transforming growth factor- β (TGF- β 1). It has also been shown that IL-1 β , IL-6 and IL-8 expression by gingival fibroblasts are stimulated by periodontopathogenic bacteria and their components (Takada *et al.*, 1991, Dongari-Bagtzoglou and Ebersole, 1996).

The human β -defensins are antimicrobial peptides that are components of the innate immune response; they are widely expressed in epithelia and, as such, are proposed to have

a role in mucosal defence. It may be that the absence or inactivation of antimicrobial peptides leads to diseases like periodontitis or opportunistic infections.

When examined by RT-PCR, h β D-1 mRNA was found to be expressed in gingiva, the parotid gland, buccal mucosa and the tongue (Mathews *et al.*, 1999). Whilst h β D-1 shows a widespread distribution in oral tissue, expression of h β D-2 mRNA was detected only in the gingival mucosa and was most abundant in tissue with associated inflammation (Mathews *et al.*, 1999). The relative expression of h β D-1 mRNA has been shown to be similar in non-inflamed and inflamed tissue from patients undergoing periodontal treatment. In normal tissue, the relative expression of h β D-1 mRNA varies between samples and was found to be unrelated to IL-8 mRNA levels (Krisanaprakornkit *et al.*, 1998). The bovine homologs TAP and LAP are expressed in bovine tongue epithelia and show a marked induction of mRNA expression in epithelia surrounding areas of inflammation (Schonwetter *et al.*, 1995). Like h β D-2 mRNA, the expression of LAP is induced in part, by bacterial LPS and proinflammatory cytokines (Diamond *et al.*, 1996).

Until now, little attention has been paid to the possible role of human defensins in the innate host defence of the oral cavity. However, reports published early in our investigations provided the first limited evidence for the importance of some human defensins in maintaining oral health (Krisanaprakornkit *et al.*, 1998 and Mathews *et al.*, 1999). To understand in more detail the relationship between tissue pathology and β -defensin synthesis, the localisation of h β D-1 and h β D-2 mRNA and protein have been determined.

3.2 Aims

- To determine the mRNA profile of human β -defensins and early response cytokines in healthy gingival tissue and gingival tissue from patients with localised aggressive periodontitis (LAP) and chronic periodontal disease.
- To localise the human β -defensins in gingival sections from healthy patients and those with adult periodontal disease.

3.3 Materials and methods

3.3.1 Patient groups

Samples of gingival tissue from patients with LAP were collected by Steven Smith, Department of Periodontology at the Eastman Dental Hospital. These were subsequently processed by Dr Peter Tabona, Dept of Microbial Diseases, Eastman Dental Institute.

The patient profile of the localised aggressive periodontitis study group was as follows:

A total of 17 patients referred to the Department of Periodontology at the Eastman Dental Institute and Hospital formed 2 groups and were included in the study. Group 1 comprised 11 patients: 6 male, 5 female, 23-35 years of age, (mean 31.3+/-years) whose clinical and radiographic presentation were consistent with the criteria so that they could be diagnosed as suffering from LAP. The criteria for LAP included, only affecting patients under 35 years of age, pronounced episodic nature of loss of attachment and alveolar bone, also the involvement of the first molar and incisor teeth. Group 2 comprised 6 patients, 3 male, 3 female, 28-59 years of age, (mean 41.8+/_years) previously diagnosed with moderate to advanced tooth wear (Smith and Knight 1984) and referred for crown lengthening surgery. Examination showed there to be a generally healthy periodontium with no periodontal pocket probing depths in excess of 3mm and without radiographic evidence of normal alveolar bone loss. They were assessed as not having periodontitis and were assigned to the control group. The patients were fit and healthy with unremarkable medical histories and

had not received antimicrobial therapy in the previous 6 months. The study had received the prior approval of the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital.

All the patients included in this study had the nature of the investigation explained to them and voluntary verbal and written consent was obtained. They were then enrolled on individual courses of prescribed initial therapy which included assessment and adjustment of their plaque control techniques, superficial scaling and polishing of standing teeth, indicated adjustment of restorative margins and, where necessary, subgingival debridement using hand and ultra-sonic instruments. Following reassessment, periodontal surgical pocket elimination procedures were planned for 17 sites in the 11 group 1 patients and periodontal surgical crown lengthening procedures for 8 sites in the group 2 patients.

Periodontal surgery was carried out by one operator (Steven Smith, Department of Periodontology, Eastman Dental Institute). Following a Modified Widman, incision the periodontal tissue biopsies obtained from the 17 sites in Group 1 and the 8 sites in Group 2 were immediately placed in a 7ml bijoux container (Bibby Sterilin Ltd, Stone, Staffordshire, England.) and snap-frozen in a Dewar flask containing liquid nitrogen, before being transferred to a sealed container in a -70°C freezer for storage prior to tissue preparation.

Gingival tissue samples from patients with periodontal disease were collected within 2 hours of surgical removal and transported to the laboratory. The patients were diagnosed as suffering from chronic periodontitis.

Surgical procedures were performed on patients attending the Department of Periodontology, Eastman Dental Hospital, as part of their normal treatment procedure. As these tissues would otherwise be discarded, at the time of investigation no ethical permission was required for this study. The group comprised 23 patients, 11 male, 12 female, 17-53 years of age, (mean 41.8 +/- years).

The distinction between non-inflamed and inflamed tissues was made on clinical evaluation using generally-accepted definitions. As opposed to healthy gingiva (no redness, no bleeding on probing and no attachment loss), samples of gingivae from patients with plaque-related gingivitis were taken from areas that showed redness and/or bleeding on probing but no clinical attachment loss. In areas of periodontitis, the tissues showed bleeding on probing, had a probing depth >5mm and clinical attachment loss.

Specimens from the diseased group were collected fresh in tissue culture medium and immediately transferred to the laboratory. Portions of each specimen were snap-frozen in liquid nitrogen for RT-PCR, and fixed in ice-cold 4% paraformaldehyde before being embedded in paraffin wax for immunocytochemical studies.

Normal gingival tissue for immunocytochemistry was obtained from tissue archives embedded in paraffin wax in the Department of Oral Pathology, Eastman Dental Institute.

From a separate frozen supply, normal tissue to be used for analysis by RT-PCR was taken; the tissue collection had been stored at -70°C in the Dept of Oral Pathology, Eastman Dental Institute. Tissue had been mounted for sectioning and stored frozen at -70°C after snap-freezing in liquid nitrogen. Using a cryostat, 10 sections were cut from tissue samples using an RNase-treated blade, and placed into a pyrogen-free 1.5ml eppendorf tube. RNA was then extracted as previously described for tissue, see chapter 2, section 2.4, except that solution D was added directly to the tube and the tissue minced using a pyrogen-free Eppendorf pestle.

3.3.2 RT-PCR

Tissue samples were prepared as described above. Total cellular RNA was extracted and 5 μg reverse transcribed, as described in Chapter 2 sections 2.4 and 2.5. PCR was performed to detect transcripts for GAPDH, h β D-1, h β D-2, IL-6, IL-1 β , IL-8, IFN- γ and IL-10, see Chapter 2, section 2.6. Primers and program details are shown in Chapter 2,

Table 2. Cycle numbers were 27 for GAPDH and 35 for all other transcripts. PCR products were run on agarose gels and visualised as described in Chapter 2, section 2.7.

3.3.3 Immunocytochemistry

Normal tissue samples for immunocytochemistry, bearing no relation to tissue samples used in RT-PCR analysis, were selected from paraffin-embedded archive material stored in the Department of Oral Pathology, Eastman Dental Institute, London, UK. Procedures are described in Chapter 2, section 2.8. Briefly, tissue samples from the adult periodontitis group were removed from 4% paraformaldehyde, orientated into moulding cassettes and placed into the citadel 1000 tissue processor for automatic embedding into paraffin wax. This involves dehydration (with increasing ethanol), clearing (with toluene), and paraffin infiltration of the tissue.

Tissue sections of 5µm thickness were cut, using a microtome and heat-fixed onto APES coated glass slides. Slides were then stained for hβD-1 and hβD-2 using pre-immune serum as a negative control, as described in Chapter 2, section 2.8.

Slides were visualised under a light microscope at a magnification of x10 or x20 and the image captured using a Polaroid digital microscope camera.

3.4 Results

3.4.1 Presence of cytokine and antimicrobial peptide mRNA in gingival samples from LAP patients

Gingival samples were obtained as described and their RNA extracted and reverse transcribed. The resulting cDNA was subjected to PCR using primers to detect transcripts for the housekeeping gene GAPDH and for h β D-1, h β D-2, IL-1 β , IL-6, IL-8, IL-10 and IFN- γ . Results for normal healthy gingivae can be seen in Table 3.1 and results for tissue from LAP patients are shown in Table 3.2, both are represented graphically as % occurrence in patient group in Figure 3.1. Table 3.1 shows 15 gingival tissue samples taken from 11 patients diagnosed with LAP, tissue samples 1, 7 and 8 were all from patient 1, tissue samples 10 and 11 from patient 7 and tissue samples 12 and 13 from patient 8. Table 3.2 shows 11 healthy tissue samples taken from 6 patients, tissue samples 17 and 18 were from patient 2, tissue samples 19, 20 and 21 were from patient 3, tissue samples 22 and 23 were from patient 4 and tissue samples 24 and 25 were from patient 5. In all samples from LAP patients and the tissue control patients, mRNA for the housekeeping gene GAPDH was detected. h β D-1 mRNA was detected in 7/15 (47%) of diseased patients compared to 1/11 (9%) in control patients. h β D-2 mRNA was detected in 10/15 (67%) of diseased patients compared to 3/11 (27%) in control patients. IL-1 β mRNA was detected in 11/15 (73%) of diseased patients compared to 3/11 (27%) in control patients, IL-6 mRNA was detected in 6/15 (40%) of diseased patients compared to 0/11 (0%) in control patients. IL-10 mRNA was detected in 3/15 (20%) of diseased patients compared to 2/11 (18%) in control patients. IFN- γ mRNA was detected in 6/15 (40%) diseased patients compared to 3/11 (27%) of control patients. IL-8 mRNA was detected in 12/15 (80%) diseased patients compared to 9/11 (82%) of control patients. One patient sample in the LAP group expressed mRNA for all pro-inflammatory and anti-inflammatory mediators. Statistical analysis using chi-squared showed there to be a significant increase in the expression of

IL-1 β mRNA ($p<0.03$) and IL-6 mRNA ($p<0.02$) in diseased tissue compared to healthy tissues.

Patient No.	Diagnosis	Sample number	GAPDH	h β D-1	h β D-2	IL-1 β	IL-6	IL-10	IFN- γ	IL-8
1 [#]	R+	1	++	-	+	+	-	-	+	+
2	J+	2	++	++	+++	-	-	+	-	+
3	R+	3	++	++	-	+	-	-	+	+
4	J+	4	++	++	+++	+	+	+	+	+
5	J+	5	++	-	++	+	-	-	-	+
1 [#]	R	7	++	+	-	+	+	+	+	-
1 [#]	R	8	++	-	-	-	+	-	-	+
6	J	9	++	+	-	+	+	-	+	++
7 [~]	R	10	++	++	++	+	+	-	-	+
7 [~]	R	11	++	++	++	+	+	-	-	+
8 [^]	J	12	++	-	++	+	-	-	-	++
8 [^]	J	13	++	-	++	+	-	-	-	++
9	R	26	++	++	++	-	-	-	+	-
10	R	27	++	-	+++	+	-	-	-	-
11	R	28	++	++	+++	-	-	-	-	+
Total			15/15	9/15	11/15	11/15	6/15	3/15	6/15	12/15
%			100	60	73	73*	40*	20	40	80

Table 3.1 Presence of mRNA for h β D-1, h β D-2, IL-1 β , IL-6, IL-10, IFN- γ and IL-8 in samples from LAP patients - negative + weakly positive ++ positive +++ strongly positive
 * statistically significant compared to healthy tissue
 #, ~, ^ - samples from the same patient
 Diagnosis - R = Rapidly Progressive Periodontitis
 J = Juvenile Periodontitis

Patient No.	Sample number	GAPDH	h β D-1	h β D-2	IL-1 β	IL-6	IL-10	IFN- γ	IL-8
1	6	++	-	++	-	-	-	-	+
2 [~]	17	++	-	-	-	-	-	-	+
2 [~]	18	++	+	+	+	-	+	+	-
3 [#]	19	++	-	++	-	-	-	+	-
3 [#]	20	++	-	-	+	-	-	-	-
3 [#]	21	++	-	-	-	-	+	-	+
4 [^]	22	++	++	+++	+	-	-	-	+
4 [^]	23	++	+	-	-	-	-	+	-
5 ^{>}	24	++	-	+	-	-	-	-	+
5 ^{>}	25	++	-	++	-	-	-	-	-
6	29	++	-	-	-	-	-	-	++
Total		11/11	3/11	6/11	3/11	0/11	2/11	3/11	6/11
%		100	27	55	27	0	18	27	55

Table 3.2 Presence of mRNA for h β D-1, h β D-2, IL-1 β , IL-6, IL-10, IFN- γ and IL-8 in samples from control patients - negative + weakly positive ++ positive +++ strongly positive
 #, ~, ^, > - samples from the same patient

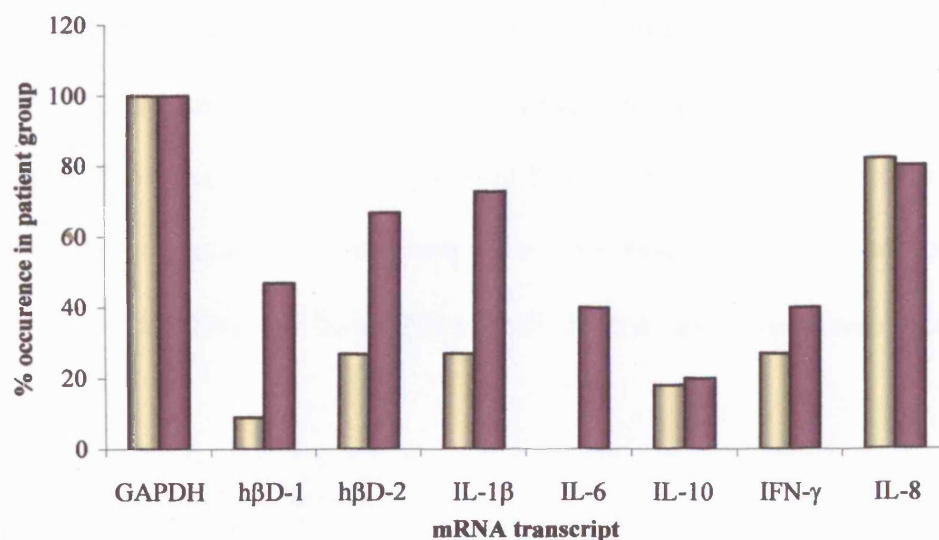


Figure 3.1 Percentage expression of cytokine and antimicrobial peptide mRNA in LAP patients
 ■ control - healthy patients ■ LAP patients

3.4.2 Presence of cytokine and antimicrobial peptide mRNA in gingival samples from chronic periodontitis patients

Gingival samples were obtained as described and their RNA extracted and reverse transcribed. The resulting cDNA was subjected to PCR using primers to detect transcripts for the housekeeping gene GAPDH and for hβD-1, hβD-2, IL-1β, IL-6, IL-8, IL-10 and IFN-γ. Results for normal healthy gingivae can be seen in Table 3.3 and results for tissue from chronic adult periodontitis patients are shown in Table 3.2, both are represented graphically as % occurrence in patient group in Figure 3.2.

In all samples from the chronic periodontitis patients and the healthy tissue control patients, mRNA for the housekeeping gene GAPDH was detected. hβD-1 mRNA was detected in 9/14 (64%) diseased patients compared to 1/12 (8.3%) in control patients. hβD-2 mRNA was detected in 12/14 (86%) diseased patients compared to 10/12 (83.3%) in control patients. IL-1β mRNA was detected in 2/14 (14%) diseased patients compared to

0/12 (0%) in control patients. IL-6 mRNA was detected in 8/14 (57%) diseased patients compared to 4/12 (33.3%) in control patients. IL-10 mRNA was not detected in any diseased patients and healthy control tissue. IFN- γ mRNA was detected in 1/14 (7%) diseased patients compared to 1/12 (8.3%) of control patients. IL-8 mRNA was detected in 14/14 (100%) diseased patients compared to 8/12 (66.6%) of control patients. Statistical analysis using chi-squared showed there to be a significant increase in the expression of h β D-1 mRNA ($p < 0.04$) and IL-8 mRNA ($p < 0.02$) in diseased tissue compared to healthy tissues.

Patient No.	Diagnosis	Sex and age	GAPDH	h β D-1	h β D-2	IL-1 β	IL-6	IL-10	IFN- γ	IL-8
2	CP	M - 45	+	++	-	-	-	-	-	+
6	CP	M - 53	+++	++	+++	-	++	-	-	++
8	CP	M - 45	+++	-	+++	+	+	-	+	++
9	CP		+++	++	++	-	+	-	-	++
12	CP	M - 50	+	-	-	-	+	-	-	+
16	CP	M - 46	++	-	+++	-	+	-	-	+
18	CP	F - 49	+	-	+	-	-	-	-	+
22	CP	M	++	+	++	+	-	-	-	+
23	CP	F - 54	+	-	+	-	-	-	-	+
25	CP	F - 38	++	+	+	-	+	-	-	+
26	CP		++	+	+	-	-	-	-	+
27	CP		++	+	+	-	+	-	-	++
29	CP	F - 46	++	+	+	-	-	-	-	++
30	CP	F - 41	++	+	++	-	+	-	-	++
Total			23/14	9/14	12/14	2/14	8/14	0/14	1/14	14/14
%			100	64*	86	14	57	0	7	100*

Table 3.3 Presence of mRNA for h β D-1, h β D-2, IL-1 β , IL-6, IL-10, IFN- γ and IL-8 in samples from chronic chronic periodontitis patients - negative + weakly positive ++ positive +++ strongly positive

* statistically significant compared to healthy tissue CP - chronic periodontitis

Sample number	GAPDH	hβD-1	hβD-2	IL-1β	IL-6	IL-10	IFN-γ	IL-8
97/1	++	-	++	-	-	-	+	+
99/14	++	-	-	-	-	-	-	+
97/2	++	-	-	-	-	-	-	-
97/3	++	-	+	-	-	-	-	+
97/9	+++	-	+	-	+	-	-	++
97/11	+++	+	+++	-	+	-	-	+
97/13	+	-	+	-	-	-	-	+
97/14	+++	-	+++	-	+	-	-	+
97/20	+++	-	++	-	-	-	-	++
97/21	+++	-	+++	-	+	-	-	-
97/22	++	-	++	-	-	-	-	-
97/37	+	-	++	-	-	-	-	-
Total	12/12	1/12	10/12	0/12	4/12	0/12	1/12	8/12
%	100	8.3	83.3	0	33.3	0	8.3	66.6

Table 3.4 Presence of mRNA for hβD-1, hβD-2, IL-1β, IL-6, IL-10, IFN-γ and IL-8 in samples from control patients - negative + weakly positive ++ positive +++ strongly positive

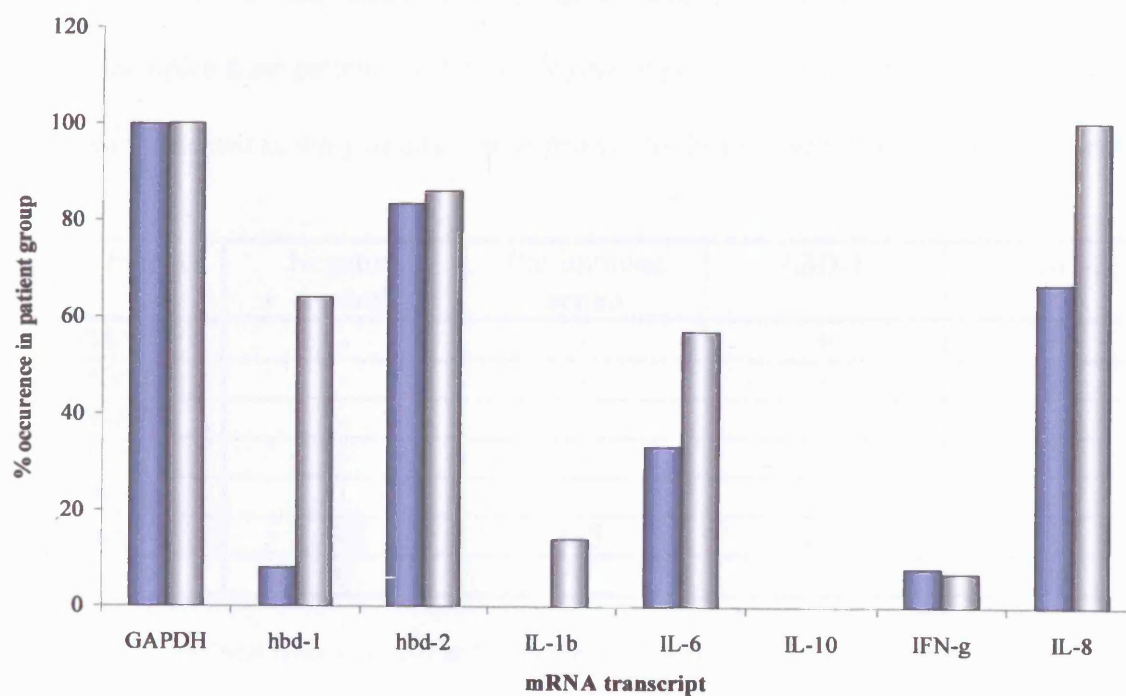


Figure 3.2 Percentage expression of cytokine and antimicrobial peptide mRNA in chronic periodontitis patients

■ control – healthy patients ■ patients with chronic periodontitis

3.4.3 Immunolocalisation of hβD-1 and hβD-2 in gingival tissue sections from adults with chronic adult periodontitis

Gingival tissue samples from adults with chronic adult periodontitis were obtained from the Department of Periodontology. Samples were divided with a portion used for RNA extraction, a portion mounted and frozen in liquid nitrogen and the final portion was fixed in 4% paraformaldehyde before mounting in paraffin wax. From the paraffin embedded samples, sections were cut and hβD-1 and hβD-2 localised in the tissue as described. Normal gingival tissue was taken from the paraffin-embedded tissue archive in Oral Pathology, sections were cut and hβD-1 and hβD-2 localised in the tissue as described.

Results for normal tissue can be seen in Table 3.5 and results for tissue from chronic periodontitis patients can be seen in Table 3.6. Antigen hβD-1 was detected in 2/5 gingival samples and hβD-2 was found in 4/5 gingival samples, taken from healthy patients. In gingival samples from patients suffering varying degrees of periodontal disease, protein for hβD-1 was detected in 4/6 patients, whilst protein for hβD-2 was detected in 3/6 patients.

Sample number	Negative control	Pre-immune serum	hβD-1	hβD-2
1684/97	-	-	+	++
1080/96	-	-	-	-
1189/95	-	-	-	+
364/95	-	-	+	+
1699/96	-	-	-	+
Total	0/5	0/5	2/5	4/5
% positive	0	0	40	80

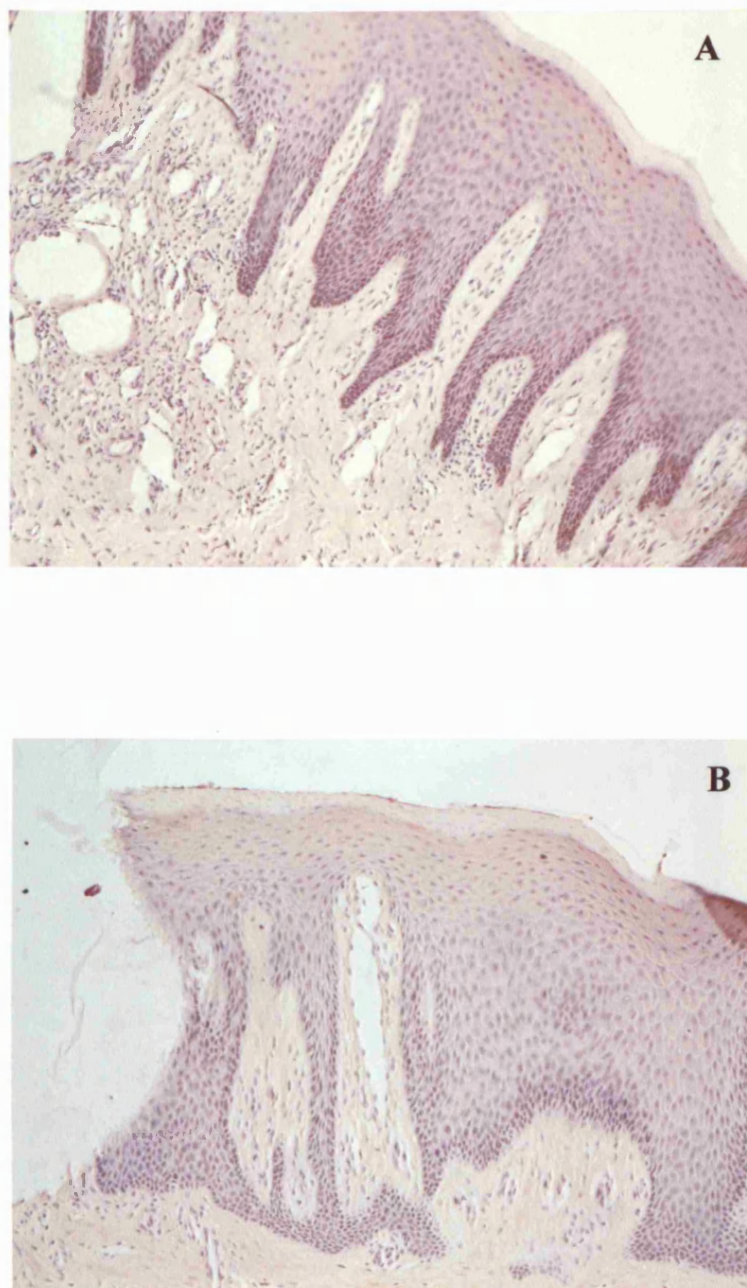
Table 3.5 Immunolocalisation of hβD-1 and hβD-2 in paraffin-embedded normal gingival samples

Sample number	Clinical diagnosis	Negative control	Pre-immune serum	h β D-1	h β D-2
6	CAP	-	-	-	-
9	CAP	-	-	-	-
10	chronic gingivitis	-	-	+	-
12	CAP	-	-	+	+
27	CAP	-	-	+	++
28	marginal gingivitis	-	-	++	++
Total		0/6	0/6	4/6	3/6
% positive		0	0	66.6	50

Table 3.6 Immunolocalisation of h β D-1 and h β D-2 in paraffin-embedded gingival samples from patients with periodontal disease

Figures 3.3 to 3.6 show immunolocalisation of h β D-1 and h β D-2 in gingival tissues. Diseased tissue taken from patient 6 (Figure 3.5) and healthy tissue from patient 1080/96 (Figure 3.4) showed no staining for either β -defensin. Gingival tissue from patient 1684/97 (Figure 3.3), diagnosed as healthy tissue showed strong staining for both β -defensins, see pages 107-108. Staining was most abundant in the uppermost layers of the epithelium, particularly in the granular layer of the epithelium rather than the cornified layer, this was more obvious for h β D-2. However both peptides were present throughout the epithelial layer and this continued into the lamina propria. Staining was not as strong for both peptides in the lower epithelial layers, the lower prickle cell layer and the basal cell layer. Gingival tissue from patient 28, diagnosed with marginal gingivitis (Figure 3.6) see pages 113-114, showed a similar pattern of expression for h β D-1 and h β D-2 protein. Staining was most abundant in the upper granular layer of the epithelium. Whilst there was some spotty expression in the more upper prickle cell layer, the lower layer and basal cell layer showed no staining for either peptide.

Figure 3.3 Patient 1684/97

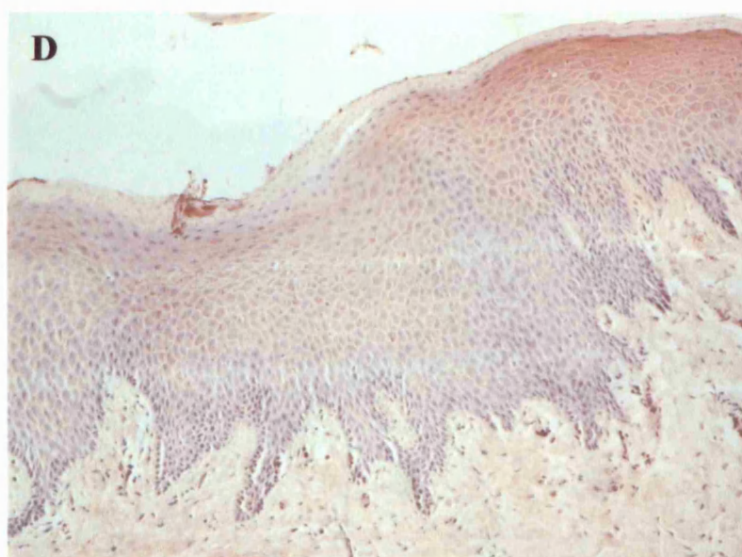
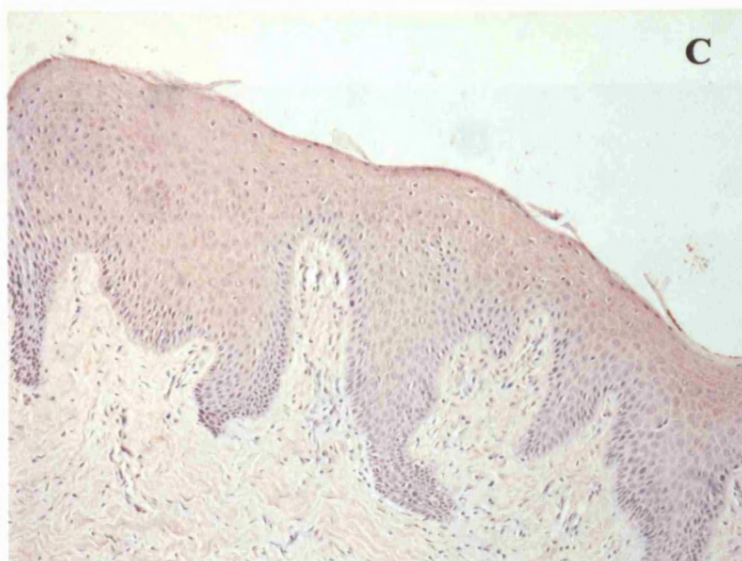


Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

A. Haematoxylin stain to show tissue morphology

B. Control pre-immune antiserum

Magnification x100



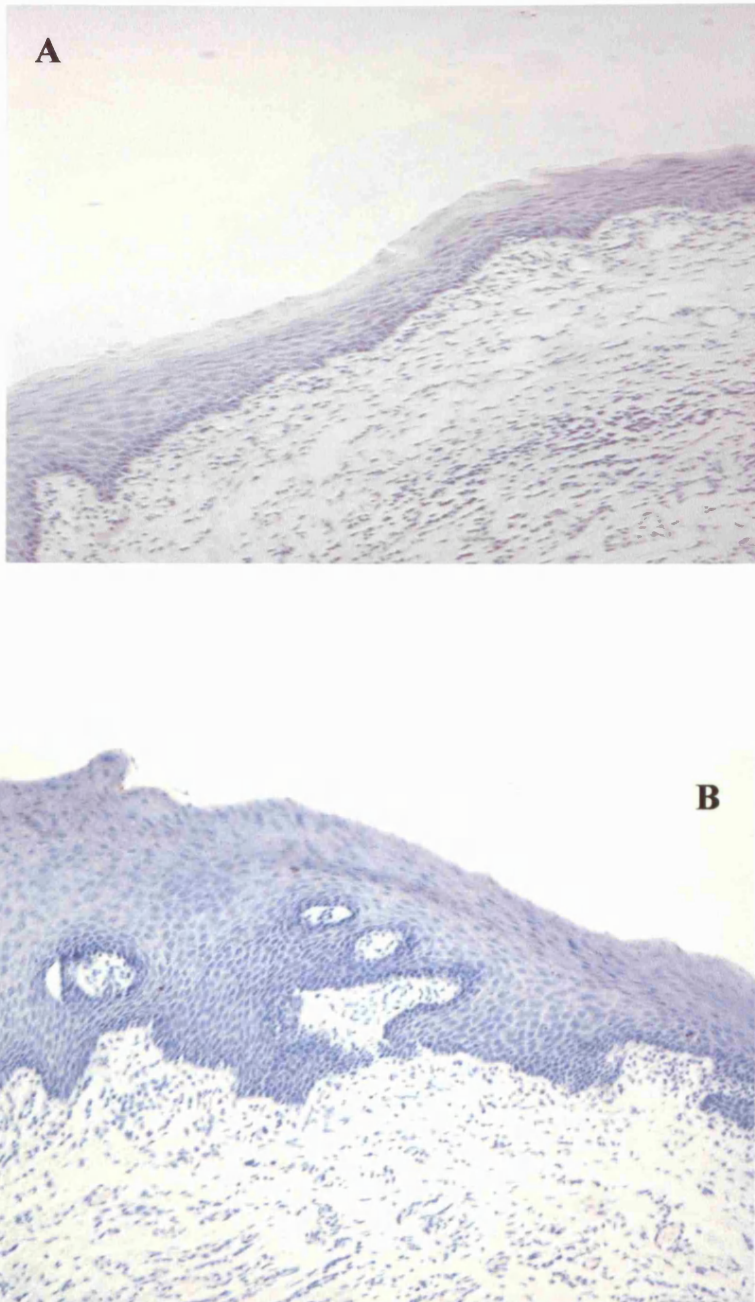
Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

C. Immunoreactivity with hβD-1 polyclonal antiserum

D. Immunoreactivity with hβD-2 polyclonal antiserum

Magnification x100

Figure 3.4 Patient 1080/96

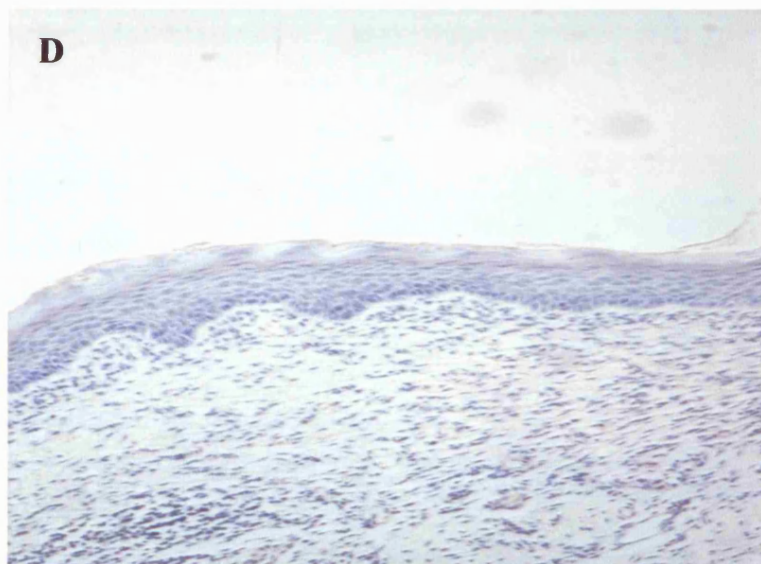


Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

A. Haematoxylin stain to show tissue morphology

B. Control pre-immune antiserum

Magnification x100



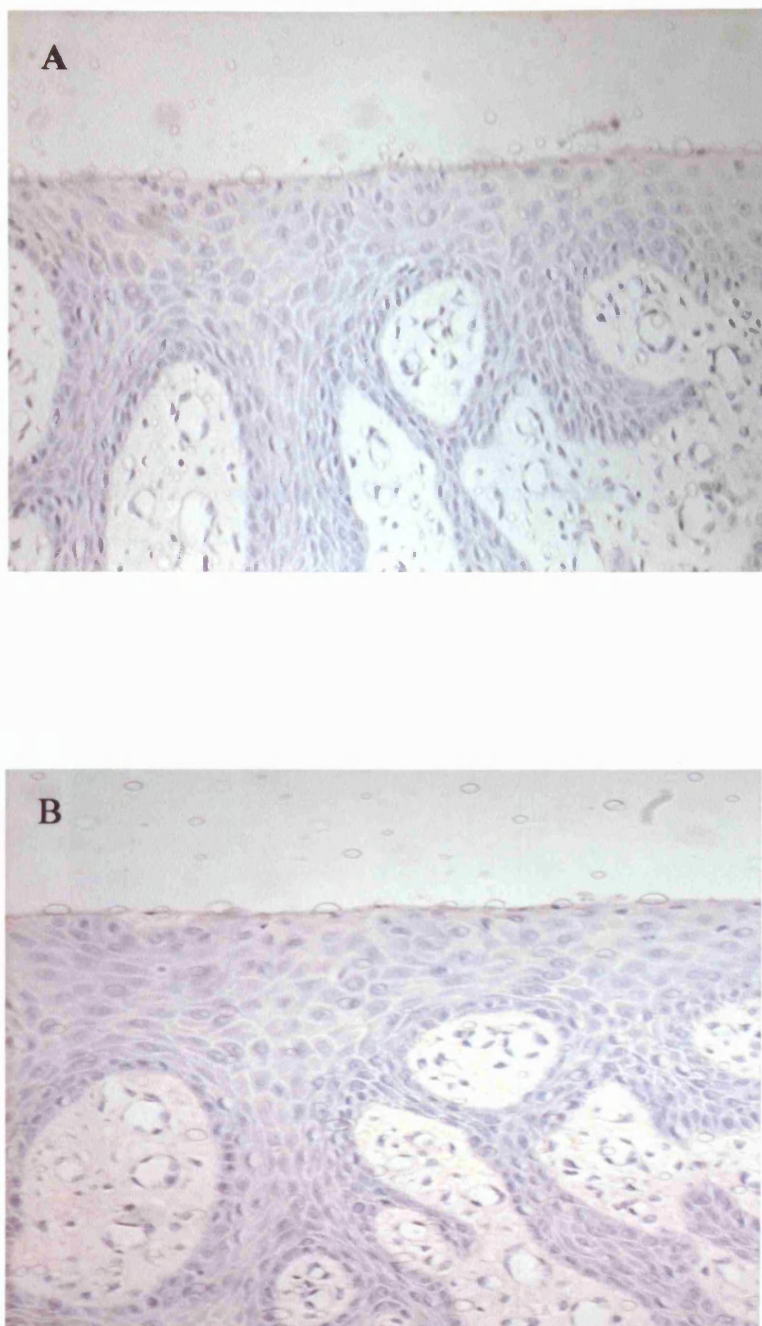
Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

C. Immunoreactivity with hβD-1 polyclonal antiserum

D. Immunoreactivity with hβD-2 polyclonal antiserum

Magnification x100

Figure 3.5 Patient 6

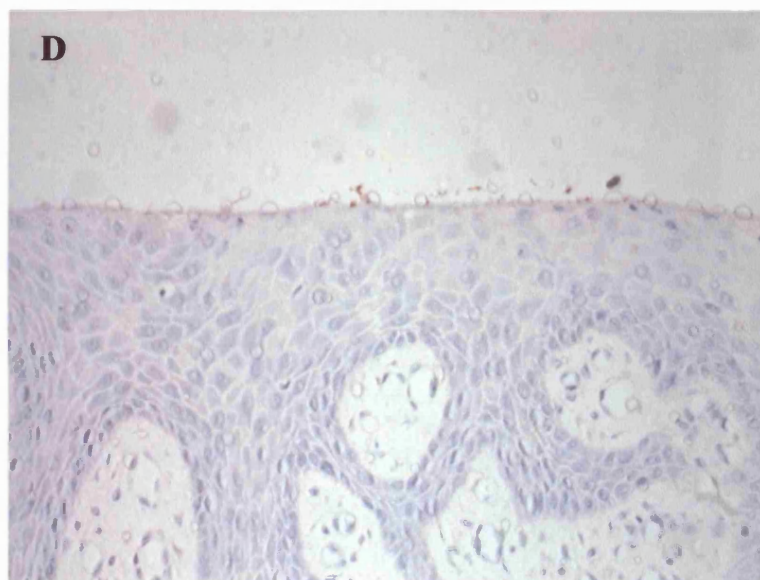
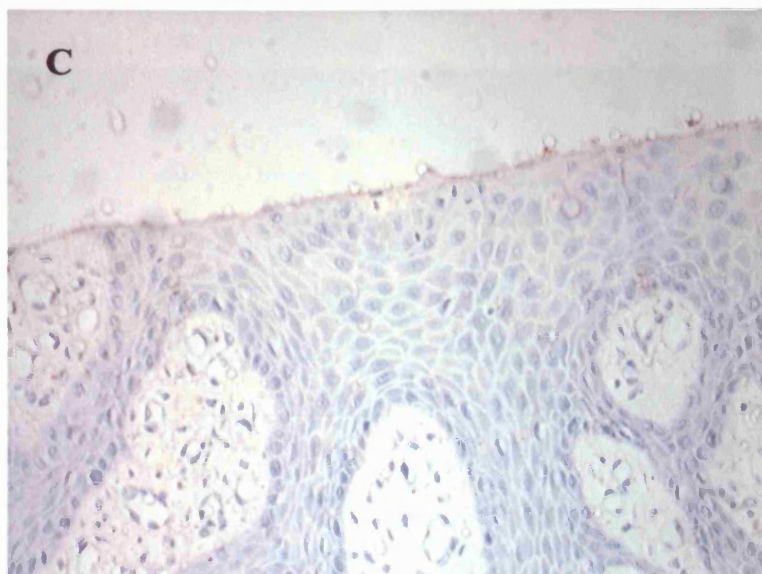


Localisation of h β D-1 and h β D-2 peptide in normal gingival oral epithelium

A. Haematoxylin stain to show tissue morphology

B. Control pre-immune antiserum

Magnification x200



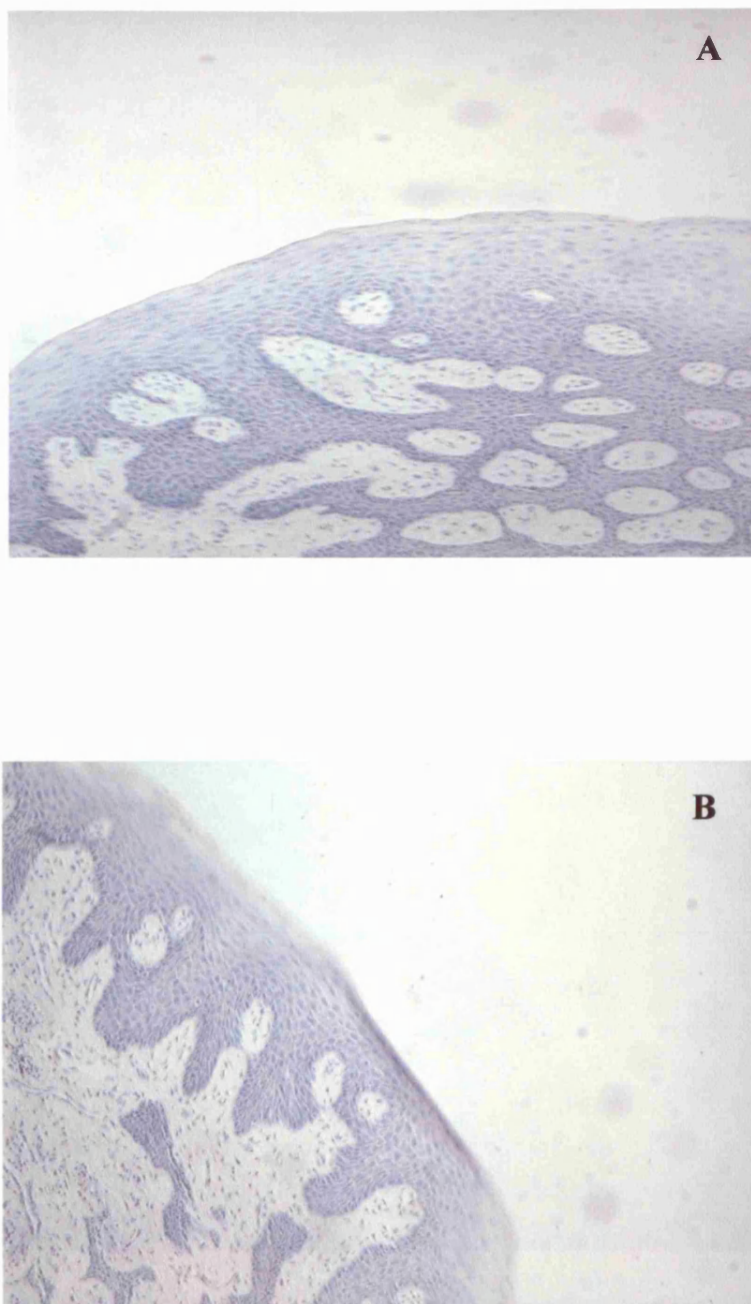
Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

C. Immunoreactivity with hβD-1 polyclonal antiserum

D. Immunoreactivity with hβD-2 polyclonal antiserum

Magnification x200

Figure 3.6 Patient 28

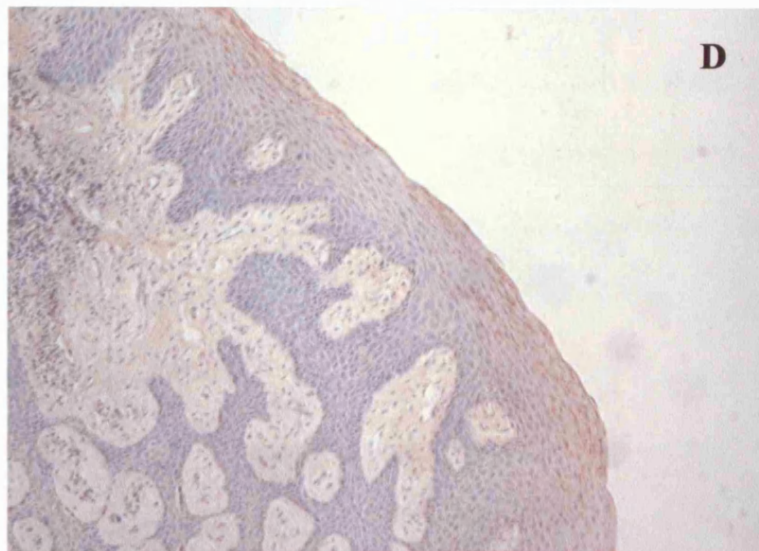
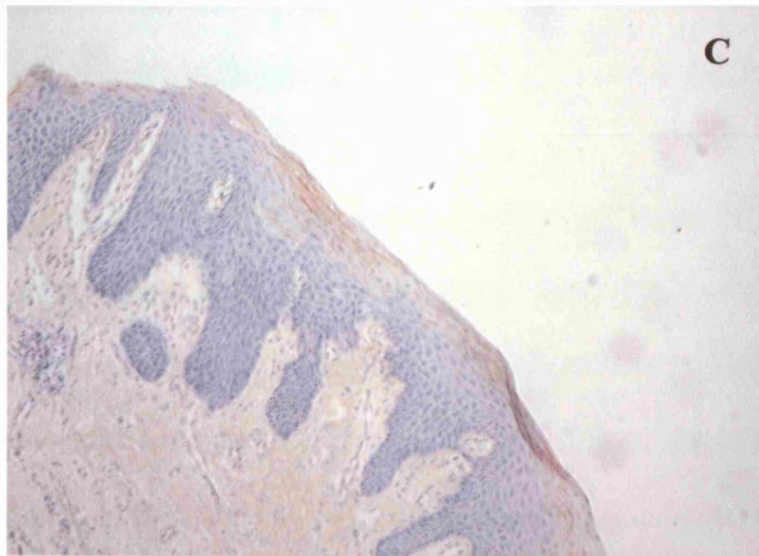


Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

A. Haematoxylin stain to show tissue morphology

B. Control pre-immune antiserum

Magnification x100



Localisation of hβD-1 and hβD-2 peptide in normal gingival oral epithelium

C. Immunoreactivity with hβD-1 polyclonal antiserum

D. Immunoreactivity with hβD-2 polyclonal antiserum

Magnification x100

3.5 Discussion

The results of studies presented in this chapter show that protein for h β D-1 and h β D-2 is expressed in gingival tissues and that expression levels are similar in healthy and diseased tissues.

Both peptides were most abundant in the uppermost-nucleated layers of the epithelium. The localisation of β -defensins in the most differentiated layers of the stratified epithelium differ from those in the pseudostratified epithelia of the trachea or the simple epithelia of the kidney in which h β D-1 is constitutively expressed and h β D-2 is inducible (Singh *et al.*, 1998 and O'Neil *et al.*, 1999). Results suggest that keratinisation in the oral epithelium plays an important role in the retention of the peptide in the epithelium and therefore its biological activity. Also, this suggests that β -defensin expression fits into the framework of the epithelial barrier and its contribution to the first line of defence.

Studies conducted at the same time as those presented in this chapter have shown that h β D-1 and h β D-2 protein can be detected by immunohistochemistry in all gingival samples tested, whether taken from clinically uninfamed or inflamed sites (Dale *et al.*, 2001). Although both peptides were not detected in all samples, the results of this study also showed there to be no difference in expression between diseased and healthy gingivae. Since the pattern of β -defensin peptide expression was found primarily in the uppermost nucleated layers of the epithelium, Dale *et al.*, 2001 suggested an association between epithelial cell differentiation and the formation of the epithelial barrier. In epithelial cell culture, h β D-1 and h β D-2 peptides were detected only in cells that were committed to differentiation, specifically those cells expressing involucrin, a differentiation marker expressed *in vivo* in suprabasal cell layers.

Dale *et al.*, 2001 also demonstrated that in normal gingival tissue, mRNAs for both h β D-1 and h β D-2 are most strongly expressed in the spinous layer of the tissue, while the peptides are detected in the upper spinous, granular and cornified layer.

Abiko and colleagues, further showed through immunostaining that the h β D-2 peptide signal was stronger in hyper-keratinised than in ortho- or non-keratinised epithelium, also signals for h β D-2 mRNA were frequently stronger in non-keratinised than in hyper- or ortho-keratinised epithelium. The authors suggest that keratinisation in the oral epithelium plays an important role in the biological function of h β D-2 both at the mRNA level and in retention of the peptide in the epithelium. Also, the non-keratinised epithelia appear to constantly express h β D-2 mRNA in order to maintain high levels of the peptide in their stratified layers to prevent bacterial infection (Abiko *et al.*, 2001, Dale *et al.*, 2001).

The results of this study prove consistent with these studies for protein expression, with strong staining for both peptides in the upper layers of the gingival epithelium.

Results also show the mRNA profiles for the human β -defensins and cytokines in healthy and diseased gingival tissues, demonstrating the different profiles between these two groups. In the LAP group there was greater expression of h β D-1, h β D-2, IL-1 β , IL-6 and IFN- γ mRNA compared to control tissues, however, only the increase in IL-1 β and IL-6 mRNA expression were found to be statistically significant. Comparing the expression of multiple immune factors also shows differences in patterns of expression. For example, the expression of both h β D-1 and h β D-2 occurs in 6 of 15 (40%) diseased tissues compared to only 2 from 11 (18%) healthy tissues. Also, in the diseased group 3 patients (20%) expressed mRNA for h β D-1, h β D-2, IL-1 β , IL-6 and IL-8 compared to none in the healthy group.

In the chronic periodontitis group there were a greater number of patients expressing h β D-1, IL-1 β , IL-6 and IL-8 mRNA compared to control patients, however, only the increased frequency of detection in h β D-1 and IL-8 mRNA was found to be statistically significant. This diseased group also displayed some patterns of expression. The expression of both

h β D-1 and h β D-2 mRNA occurred in 8 of 14 (57%) diseased tissues, compared to 1 of 12 (8.3%) healthy tissues. Also in the diseased group, 5 patients (35.7%) expressed mRNA for h β D-1, h β D-2, IL-6 and IL-8 compared to none in the healthy group.

Whilst recent reports have demonstrated the expression of human β -defensins in oral tissues, the associated cytokine profiles of such samples has yet to be reported. The results of this study have shown for the first time that there are links in the pattern of expression between h β D-1, h β D-2 and the pro-inflammatory cytokine mRNA in diseased gingival tissue. Since in approximately half of CP patients the human β -defensin h β D-1 and IL-6 are expressed at the same time, it is interesting to note that the 5'-flanking region of the h β D-1 gene contains nuclear factor (NF) IL-6 and γ -interferon consensus sites, suggesting that inflammatory mediators could regulate its expression (Valore, 1998, Van Wetering, 1999). Further it has been shown in cultured lung epithelial cells that IL-1 β and TNF- α upregulate the expression of h β D-2 mRNA (Harder *et al.*, 2000). However evidence presented in this chapter fails to support this since in the healthy CP control group 83% of patients expressed h β D-2 mRNA whilst none expressed mRNA for IL-1 β .

It is now known that h β D-2 mRNA is produced upon stimulation with exogenous stimuli such as microbial constituents or endogenous signals such as TNF- α and IL-1 β and contributes to the local host defence of human epithelia (Harder *et al.*, 1997, Bals *et al.*, 1998, Krisanaprakornkit *et al.*, 2000). It is therefore interesting to note that in the LAP group, of the 11 patients expressing IL-1 β , 8 also expressed mRNA for h β D-2. Similarly in the healthy control group, 2 of 3 patients expressing IL-1 β also expressed mRNA for h β D-2. In the chronic periodontitis group, both patients expressing mRNA for IL-1 β also expressed mRNA for h β D-2, none of the patients in the healthy control group expressed mRNA for IL-1 β . It has been shown that IL-1 β treatment of oral gingival keratinocytes results in upregulation of h β D-2 mRNA, the stimulatory effect was also shown to be

greater than that achieved by *E. coli* LPS. In contrast, h β D-1 mRNA levels were unchanged in the presence of either IL-1 β or LPS (Mathews *et al.*, 1999). It has also been recently demonstrated that h β D-2 mRNA is induced in the epidermis by LPS-treated monocyte-derived cells (MoDeC), and its induction reversed by IL-1ra. Thus, the epidermal response to LPS is potently amplified by MoDeC through IL-1-mediated signalling, leading to a selective increase in the synthesis of h β D-2 (Liu *et al.*, 2003).

The human β -defensins seem to be a poor marker for inflammation in the oral cavity as they are found in both healthy and diseased tissues. This is in contrast to the epidermis, in which h β D-2 mRNA is seen primarily in association with inflammation or disease (Harder *et al.*, 1997). It may be that h β D-1 is a constant presence in oral tissue to maintain homeostasis between the host and indigenous oral bacteria. h β D-1 has been shown to be constitutively expressed in oral keratinocytes and to be unaffected by bacterial or pro-inflammatory stimuli (Krianaprakornkit *et al.*, 1998, Mathews *et al.*, 1999).

The role of h β D-2 appears to be more complex; studies show that it is abundantly expressed in healthy tissue. This partially-stimulated state may be due to its constant exposure to oral commensal microorganisms. The unstimulated level of h β D-2 in oral keratinocytes is extremely low and upregulation of mRNA occurs rapidly (Krisanaprakornkit *et al.*, 2000). Stimulants include the oral commensal *F. nucleatum* and *P. gingivalis*, which is known to be associated with periodontal disease.

In addition to their direct antimicrobial role, it has now been recognised that defensins have important signalling potential, exhibiting cross-talk between the innate and acquired immune responses. h β D-2 has been shown to be chemotactic for immature dendritic cells (professional antigen presenting cells) and memory T-cells to activate more long-term defences (Yang *et al.*, 1999). It may be that the immature dendritic cells and memory

T-cell population of healthy subjects are more chemotactic to h β D-2 and this offers protection against the development of periodontal disease. The chemotactic effect of h β D-2 on immature DCs and memory T cells is mediated by the human CC chemokine receptor 6 (CCR6) (Yang *et al.*, 1999). Interestingly, in addition to the functional overlap between defensins and chemokines, it has recently been shown that the IFN- γ -inducible chemokines MIG/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 have antimicrobial activities against *E. coli* and *Listeria monocytogenes* (Cole *et al.*, 2001).

It may be that the infiltration of chemoattracted leukocytes at sites of periodontal inflammation could contribute to the increased presence of h β D-2 mRNA found in inflamed gingival tissue samples in this study. The results presented in Chapter 4, section 4.4.3 show that both h β D-1 and h β D-2 mRNA were present in peripheral blood mononuclear cells and that h β D-2 was upregulated by the bacterial component LPS.

These results also show differences in expression between similar groups. Of the 2 control groups of 11 and 12 healthy gingival samples, 27 and 8.3% expressed mRNA for h β D-1, while 55 and 83.3% expressed mRNA for h β D-2, suggesting variation between individuals. Studies investigating the expression of h β D-2 mRNA in human gingival epithelial cells have found conflicting expression in stimulated and unstimulated cells; one possible explanation may be that the cells are from different individuals (Abiko *et al.*, 1999, Krisanaprakornkit *et al.*, 2000). Variability of h β D-2 mRNA expression may also be present in specific groups. In a study conducted by Ali *et al.*, 2001, it was demonstrated that there is variable expression of β -defensins amongst different skin donors. While genetic differences between donor subjects may explain some variation in expression levels, the oral epithelium, like the skin, is constantly exposed to bacteria and environmental stimuli that may vary widely across and within donor populations. It is

likely that these factors also play a role in modulating the host's immune response. In the 2 groups of 15 and 23 diseased gingival samples, the levels of β -defensins were more comparable, 60 and 61% expressed mRNA for h β D-1, while 73 and 87% expressed mRNA for h β D-2. Also, between groups the expression of the housekeeping gene was highly variable and not controlled for, therefore these results in no way provide a quantitative analysis and they purely demonstrate the presence or absence of the human β -defensins and cytokines in oral tissues.

In order to determine a definite difference, if indeed one exists in the expression of human β -defensins between healthy and inflamed oral tissue, it is obvious that a greater number of samples need to be examined and subjected to analysis using RT-PCR, immunocytochemistry and *in situ* hybridisation.

These findings, which demonstrate increased expression of IL-6 and IL-1 β in the early onset periodontitis group, are in agreement with previous studies (Masada *et al.*, 1990 Stashenko *et al.*, 1991, Tsai *et al.*, 1995). These latter studies have shown that IL-1, IL-6, IL-8 and TNF- α levels are increased in the periodontal tissue and/or gingival crevicular fluid from diseased sites compared to those in healthy sites. However, Lundqvist and co-workers (1994) showed that gingival epithelial cells freshly isolated from normal and inflamed gingivae expressed IL-1 β , IL-6, IL-8, TNF- α and transforming growth factor- β (TGF- β 1) and that the cytokine profiles of epithelial cells from normal and inflamed gingivae were similar. Since only gingival epithelial cells were examined, this study does not take into account the expression of cytokines in the invading lymphocytes in periodontal lesions and therefore may not be representative of periodontal inflammation.

In this study only limited data on the lifestyle and medical history of the patients included in the study were available, these details may have further explained the cytokine profiles

found. Analysing cytokine profiles in individual subjects provides insight into the cytokine network controlling local periodontal tissue reactions.

The fact that cytokine mRNA levels vary considerably between individuals, and between sites within the same individual, would complicate matters for the clinician, but reflects the biological complexity of the cytokine network (Bickel *et al.*, 2001). Cytokine profiles are of considerable value when studying disease mechanisms (Kornman 1997, Okada *et al.*, 1996) but may not be suitable diagnostic markers in periodontitis.

Chapter 4

The effect of *E. coli* LPS on oral epithelial cells and PBMCs

Chapter 4

The effect of LPS on oral epithelial cells and PBMCs

4.1 Introduction

Mucosae are our first line of defense against infection; in particular the oral mucosa functions as a protective barrier against the pathogenic microorganisms present in dental plaque. It is now widely recognised that epithelial cells participate in innate immunity; which is induced when loosely-defined recognition elements of microbes bind to pattern recognition receptors present on both phagocytic and epithelial cells (Diamond *et al.*, 2000). Mucosal epithelial cells contribute to innate immunity by expressing antimicrobial peptides, including the β -defensins, h β D-1, h β D-2 and h β D-3, as well as chemokines that attract monocytes and neutrophils and cytokines which activate the adaptive immune system. Characterisation of β -defensin regulation is essential for understanding the role of these peptides in protecting the host by activating both innate and adaptive immune systems and in contributing to the epithelial barrier in inflammatory disease processes.

LPS is one of the primary stimuli of the innate element of the mammalian immune system. Recognition of LPS by cells such as monocytes and macrophages has evolved over millions of years to provide all mammalian hosts with rapid recognition of, and reaction to, Gram negative infection. The rapid innate response against LPS typically involves the release of a range of inflammatory mediators, such as TNF- α , IL-6 and IL-1 β . In local sites of infection and at moderate levels, these mediators benefit the host greatly by promoting inflammation and otherwise priming the innate immune system to eliminate the invading microorganisms.

In addition to their widespread expression by epithelial cells and their obvious role in mucosal defence, β -defensins have also been reported to be expressed by murine, bovine and porcine alveolar macrophages (Ryan *et al.*, 1998, Zhang *et al.*, 1998, Morrison *et al.*,

1998). The human α -defensins constitute approximately 25-30% of the human azurophilic granule proteins of neutrophils. The defensins HNP-1 and HNP-2 have been reported to be monocyte chemoattractants. The infiltration of leukocytes into the site of inflammation in periodontal disease is an important process following infection by micro-organisms. Because β -defensins are released upon microbial infection and are located at the host-environment interface, such as mucosal surfaces and skin, they may also function to alert the adaptive immune system of vertebrates. Yang *et al.*, 1999a demonstrated that h β D-2 is chemotactic for immature dendritic cells and memory T cells, therefore β -defensins may promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial infection. At the time of this study there were no reports of the presence of the human β -defensins in peripheral blood mononuclear cells.

Recently, studies have shown that *E. coli* LPS induction of h β D-2 in human tracheobronchial epithelial cells (hTBE) requires CD14, which may complex with a TLR to ultimately activate NF- κ B (Becker *et al.*, 2000). The expression of functional CD14 and TLR4 has been reported in normal human skin keratinocytes (Song *et al.*, 2002).

Becker *et al.*, 2000 also examined hTBE cells for the expression of TLR genes by RT-PCR. Their results show that mRNA for hTLR1-6 is expressed in hTBE cell cultures. Based on published results, TLR2 and TLR4 are probable LPS-signalling intermediates (Yang *et al.*, 1999b, Kirschning *et al.*, 1998, Chow *et al.*, 1999).

4.2 Aims

The purpose of this study was to determine the effect of *E. coli* LPS on the production of IL-8, h β D-2, SLPI, TLR2 and TLR4 by an oral epithelial cell line. The potential role of CD14 in the upregulation of IL-8 and h β D-2 in oral epithelial cells was then determined.

In addition, since there had been no previous reports on the presence of the human β -defensins in peripheral blood mononuclear cells, their presence in these cells, and the influence of *E. coli* LPS on their expression, was determined.

4.3 Materials and methods

4.3.1 Stimulation of the oral epithelial cell line H357

The oral epithelial cell line H357 was used to determine the effect of LPS on oral epithelial cells. For the purposes of all the experiments, *E. coli* LPS, 055:B5 (Becton Dickinson, Oxford, UK) was used. Cells were maintained as in Chapter 2 section 2.2.1. For the purposes of these experiments, cells were prepared and treated as follows.

To T75 flasks confluent with H357 cells, 1ml of trypsin-EDTA (0.25%) was added and incubated at 37°C for 10 minutes until cells had detached from the surface. After addition of fresh medium, cells were centrifuged at 1500rpm for 5 minutes and resuspended in 10ml of fresh medium. Cells were counted using a haemocytometer, diluted accordingly with medium and seeded into 6-well tissue culture plates to achieve a density of 125,000 cells/well in 3ml of medium. Tissue culture plates were then incubated at 37°C in air/5% CO₂ for approximately 40 hours until cells were 80% confluent. Monolayers were then washed with PBS and 3ml of fresh medium added. LPS diluted in PBS was added to cell monolayers at a final concentration of 100ng/ml or 1 μ g/ml, in triplicate. Cell monolayers were then incubated for the times indicated in the figures. After incubation, 1ml of medium was removed, placed in a 1.5ml eppendorf and frozen at -70°C. At a later date, an IL-8 ELISA, as described in Chapter 2 section 2.3, was carried out. The remaining medium was

removed from the cells, which were then washed with PBS. Monolayers were then lysed by the addition of 600µl Solution D and the plates stored at -70°C until RNA was extracted and analysed by RT-PCR as detailed in Chapter 2, section 2.4 - 2.6. PCR for GAPDH was for 26 cycles and those for hβD-2, IL-8, SLPI, TLR2 and TLR4 were for 35 cycles, procedures, primers and programs were as described in Chapter 2, Table 2. 20µl of PCR product was loaded and run on a 2% agarose gel as described in Chapter 2 section 2.7.

4.3.2 Stimulation of PBMCs with 1ng/ml LPS

PBMCs were prepared as described in Chapter 2, section 2.2.2. Cells were seeded into 6-well plates at a density of 2 million cells per ml and left for 2 hours to adhere. Cells were then washed and fresh medium added. Cells were stimulated with 1ng/ml LPS for 2, 4 and 18h. After each time period, the supernatant was removed and cells were washed with PBS. RNA was then extracted and RT-PCR carried out on all samples, this was as described in Chapter 2, sections 2.4 – 2.6. Results show PCR for GAPDH, 26 cycles, hβD-1, 35 cycles and hβD-2, 35 cycles, primers and programs were as described in Chapter 2, Table 2. 20µl of PCR product was then loaded and run on a 2% agarose gel, as described in Chapter 2, section 2.7.

4.3.3 FACS analysis of cell surface CD14

This was as described in Chapter 2, section 2.9. Additionally, oral epithelial H357 cells were incubated overnight with 1µg/ml LPS and PBMCs incubated overnight with 1ng/ml LPS. Data were analysed and represented as described in Chapter 2, section 2.9.1.2.

4.3.4 The effect of MY4 on LPS stimulation of the oral epithelial cell line H357

Antibodies

- MY4, a mouse monoclonal antibody to CD14 (Coulter Immunology)
- IgG2b, the antibody isotype control (Sigma, Poole, UK)

Cells were prepared as in section 4.3.1. Monolayers were then pre-incubated with the antibody MY4 and the isotype-matched control antibody IgG2b at a concentration of 5µg/ml for 1 hour. *E. coli* LPS was then added to cell cultures to a final concentration of 1µg/ml and incubated for 8h with the blocking antibody still present. Controls included cells incubated with MY4 and IgG2b at a concentration of 5µg/ml or *E. coli* LPS at a concentration of 1µg/ml. After incubation, 1ml of medium was removed, placed in a 1.5ml eppendorf and frozen at -70°C . At a later date, an IL-8 ELISA, as described in Chapter 2 section 2.3, was carried out. The remaining medium was removed from the cells, which were then washed with PBS. Monolayers were then lysed by addition of 600µl Solution D and the plates stored at -70°C until RNA was extracted and analysed by RT-PCR as detailed in section 2.4 – 2.6. PCR for GAPDH was 26 cycles and hβD-2 for 35 cycles, primers and programs for each are described in Chapter 2 Table 2. 20µl of PCR product was loaded and run on a 2% agarose gel, as described in Chapter 2, section 2.7.

4.4 Results

4.4.1 The effect of 100ng/ml *E. coli* LPS on oral epithelial cells

4.4.1.1 IL-8 production

H357 monolayers were incubated with 100ng/ml *E. coli* LPS for various times and the cell culture supernatant measured for IL-8 concentration as described in Chapter 2, section 2.3. The graph in Figure 4.1 shows the levels of IL-8, in pg/ml, secreted by stimulated oral epithelial cells. The control sample, time 0h, shows the background level of IL-8 detected in cell culture supernatants where the oral epithelial cells had not been incubated with *E. coli* LPS. After 4 hours, the level of IL-8 in both the control sample and those stimulated with 100ng/ml were no higher than was detected in the control sample, the background level. However, after 8 hours, whilst the control was still at background level, cells stimulated with 100ng/ml LPS showed a significant 7-fold ($p<0.005$) increase in the secretion of IL-8. After a 24h time period, the amount of IL-8 produced from control cells had increased dramatically from levels determined at 8h, showing a 4-fold increase. Despite this, IL-8 levels from monolayers incubated with 100ng/ml LPS for 24h had also increased and a small, 1.3-fold, but significant increase ($p<0.05$) compared to the control could be seen. After stimulation with 100ng/ml *E. coli* LPS, studies show that maximal secretion of IL-8 from oral epithelial cells can be seen at 8h with a slight decrease seen after 24h. Assays were carried out in triplicate and repeated; results shown are representative of repeated experiments.

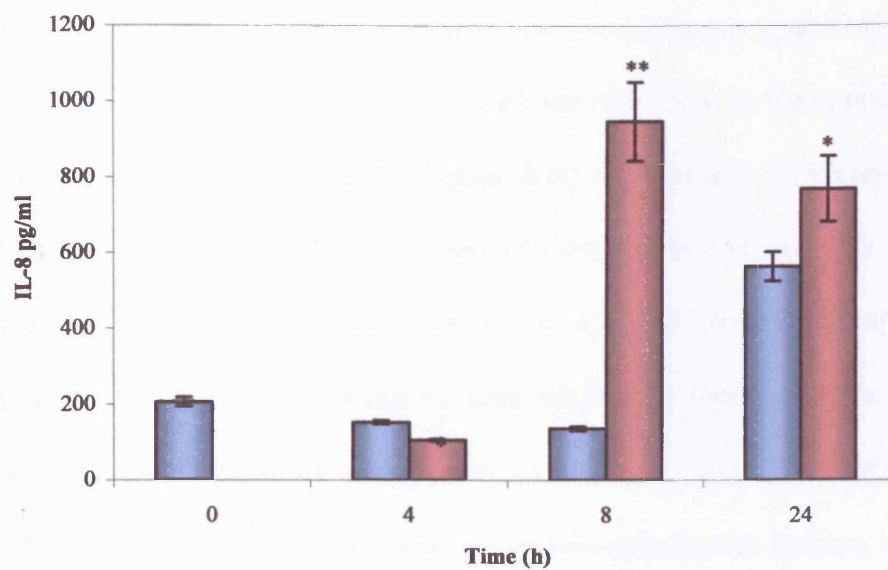


Figure 4.1 Time course of IL-8 secretion by oral epithelial cells following treatment with 100ng/ml *E. coli* LPS ■ control ■ LPS 100 ng/ml
IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures *, $P < 0.05$ **, $P < 0.005$ versus control

4.4.1.2 Messenger RNA expression

As detailed in the methods section, cell monolayers were lysed, the RNA extracted, reverse transcribed and PCR performed to detect transcripts for GAPDH, h β D-2, IL-8, SLPI, TLR2 and TLR4. Constitutive expression of the housekeeping gene GAPDH, IL-8 and SLPI, TLR2 and TLR4 mRNA was seen in all samples, both in the control samples and those stimulated with 100ng/ml LPS (Figure 4.2). A difference in expression of h β D-2 mRNA was detected between stimulated and unstimulated cell monolayers. Faint bands of h β D-2 message can be seen in the controls at 0, 4, 8 and 24h, whereas after stimulation with 100ng/ml LPS, upregulation can be seen at all time points with the most obvious difference at 8h. Slight increases were seen at 4 and 24h. As with IL-8 protein (see section 4.3.1.1) the induction of h β D-2 mRNA occurs in a time-dependent fashion, with increasing message seen up to 8 hours, where maximal induction can be seen, followed by a decrease at 24 hours. Assays were carried out in triplicate and repeated; results shown are representative of repeated experiments.

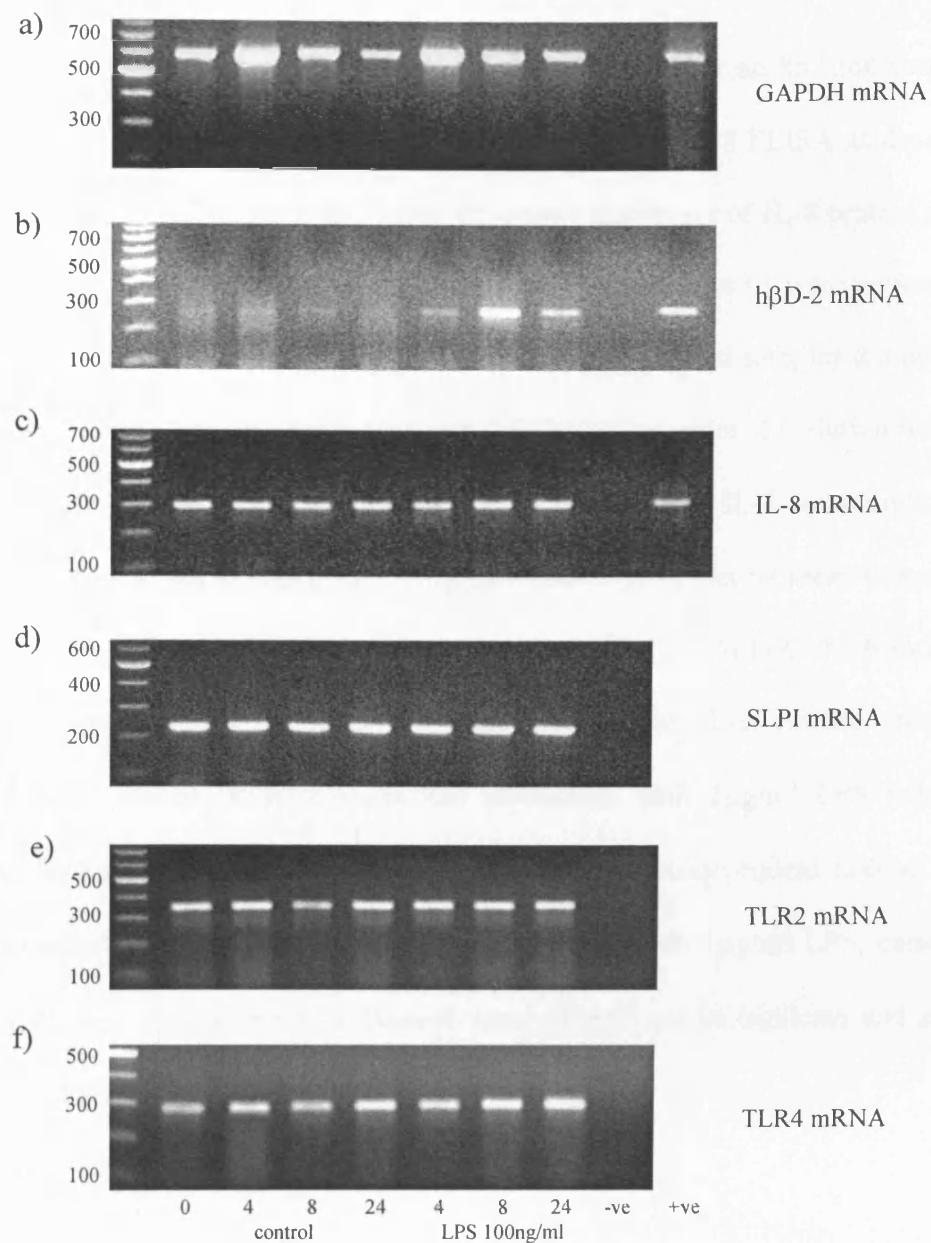


Figure 4.2 Relative levels of mRNA obtained from oral epithelial cells treated with 100ng/ml *E. coli* LPS for various times. Results show PCR products for a) the housekeeping gene GAPDH, b) hβD-2, c)IL-8, d)SLPI, e)TLR2 and f)TLR4

4.4.2 The effect of 1µg/ml of *E. coli* LPS on the oral epithelial cells

4.4.2.1 IL-8 protein

H357 monolayers were incubated with 1µg/ml *E. coli* LPS over an 8h time course with sampling at 2h intervals. Cell supernatants were analysed by IL-8 ELISA as described in Chapter 2, section 2.3. The graph in Figure 4.3 shows the levels of IL-8 protein released. As previously seen with 100ng/ml LPS, background levels (time 0) were approximately 200pg/ml. After 2h, the level of IL-8 in the control and stimulated samples did not exceed the background levels. Unlike results obtained with 100ng/ml, after stimulation for 4h with 1µg/ml *E. coli* LPS, there was an increase in the secretion of IL-8 by stimulated cells compared to controls. An approximate 2-fold increase in IL-8 can be seen; this result did not prove to be significant when analysed using Student's t-test. At both the 6 and 8h time point, significant ($p < 0.05$) 2.6 and 3.4-fold increases in IL-8 release from H357 monolayers were shown. Results show that incubation with 1µg/ml LPS induces the secretion of IL-8 from oral epithelial cells in a time- and dose-dependent fashion. Greater increases in secretion of IL-8 were seen after stimulation with 1µg/ml LPS, compared to 100ng/ml LPS (see section 4.3.1.1). Assays were carried out in triplicate and repeated; results shown are representative of repeated experiments.

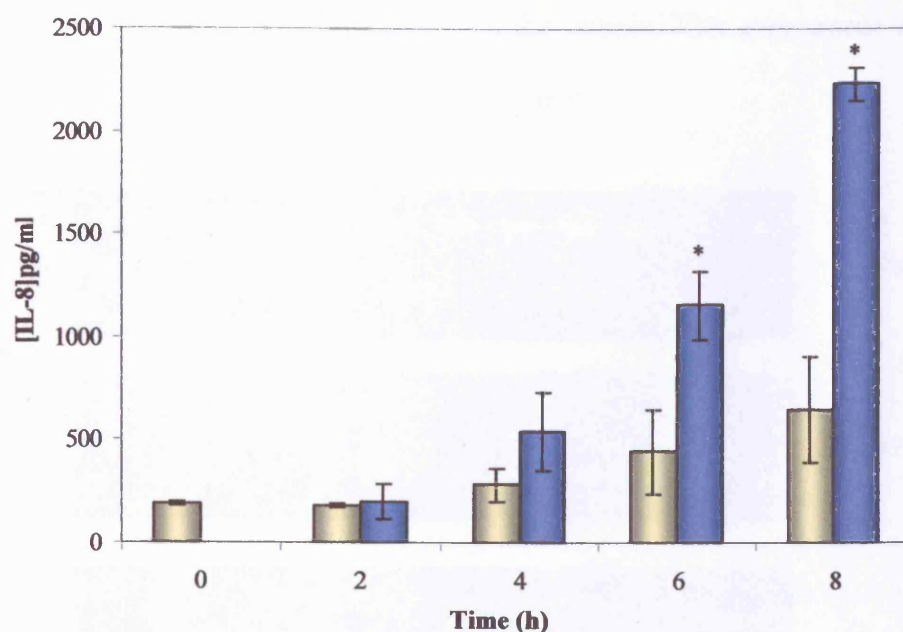


Figure 4.3 Time course of IL-8 secretion from oral epithelial cells following treatment with 1 µg/ml *E. coli* LPS. ■ control ■ LPS 1 µg/ml. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean ± standard deviation of triplicate cultures *, $P < 0.05$ versus control

4.4.2.2 Messenger RNA expression

As detailed in the methods section, cell monolayers were lysed, the RNA extracted, reverse transcribed and PCR performed to detect mRNA transcripts for GAPDH, hβD-2, IL-8, SLPI, TLR2 and TLR4. As with 100ng/ml LPS, in the control samples and those stimulated with 1 µg/ml LPS, there was constitutive expression of the housekeeping gene gapdh, IL-8, SLPI, TLR2, TLR4 and CD14 mRNA (see Figure 4.4). As when cells were stimulated with 100ng/ml LPS, the greatest difference in expression was seen with mRNA for hβD-2. Control samples showed low level expression at 0, 2, 4, 6 and 8 hours. When stimulated with 1 µg/ml LPS, hβD-2 mRNA was significantly upregulated, at all the time points, compared to the controls (Figure 4.4). Results suggest the maximal stimulation of mRNA for hβD-2 occurs after only 2 hours, since prolonged exposure does not further upregulate the production of message for hβD-2. The reduced expression of mRNA in all

of the control samples at 2h suggest problems with the methodology, some of the mRNA appears to have been lost in the processing of the sample. This experiment was repeated; results shown are representative of repeat experiments.

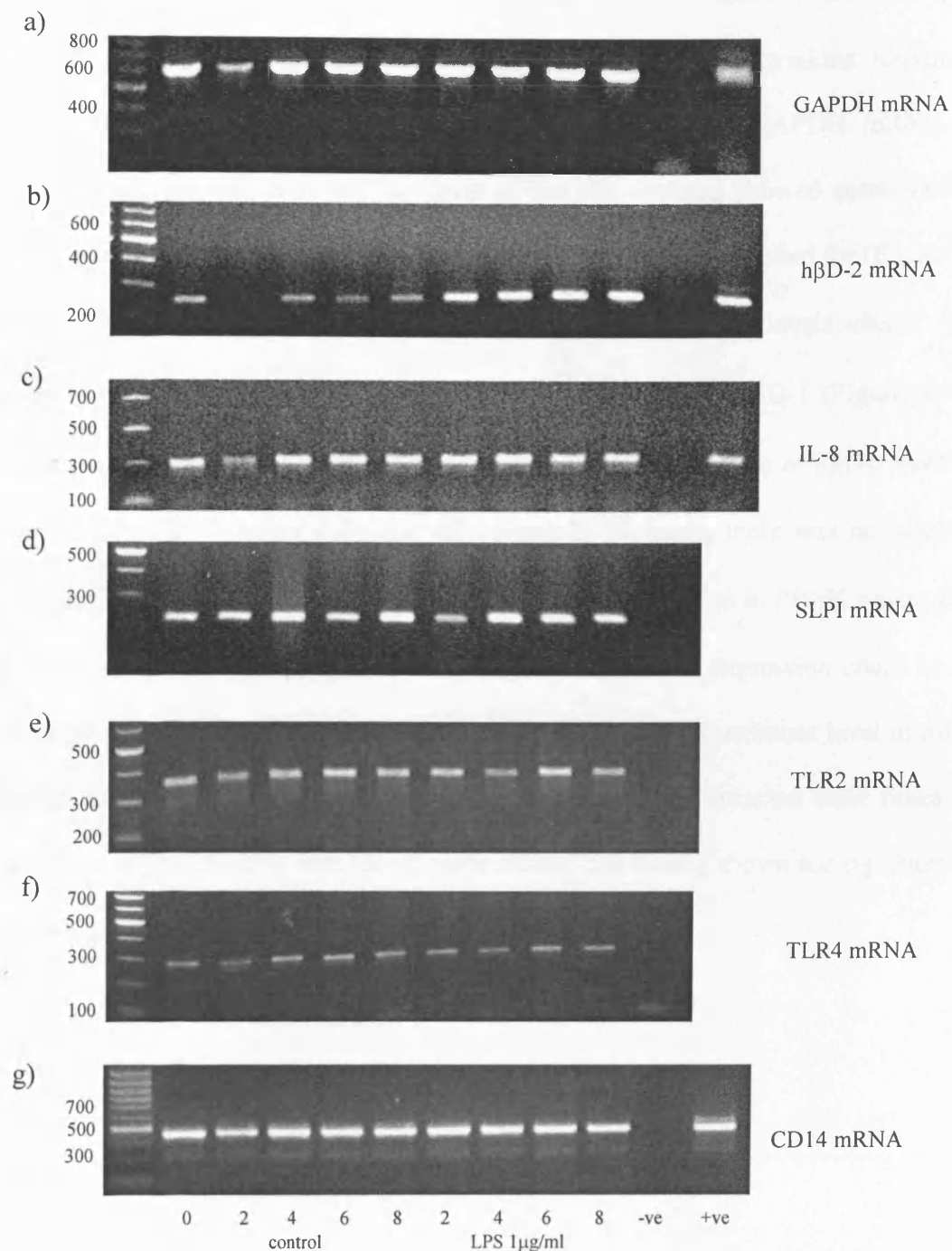


Figure 4.4 Relative levels of mRNA obtained from oral epithelial cells treated with 1μg/ml *E. coli* LPS for various times, in hours. Results show PCR products for a) the housekeeping gene GAPDH, b) hβD-2, c) IL-8, d) SLPI, e) TLR2, f) TLR4 and g) CD14

4.4.3 Expression of h β D-1 and h β D-2 mRNA in PBMC after incubation with 1ng/ml LPS

PBMCs were separated from buffy coat blood, and stimulated with LPS to determine the effect on production of mRNA for the human antimicrobial peptides, h β D-1 and h β D-2. Since lower concentrations of LPS have been shown to elicit a cytokine response in PBMCs, 1ng/ml *E. coli* LPS was used in these experiments. GAPDH mRNA was expressed in all samples; however the level of mRNA detected showed some variation since the amount of RNA used for reverse transcription was not controlled for (Figure 4.5). In non-activated monocytes, h β D-1 mRNA was expressed at low levels after 2 and 4 hours, cells incubated overnight no longer produced mRNA for h β D-1 (Figure 4.5). In cells incubated with 1ng/ml LPS, there was also a low level expression of h β D-1 mRNA at 2 hours, however by 4 hours and, like the control at 24 hours, there was no detectable message. Either no or very low levels of h β D-2 mRNA expression in PBMCs were found when these were not activated. However, a marked increase in expression could be seen after 4 hours and overnight incubation with 1ng/ml LPS, with a maximum level of mRNA expression seen at 4 hours (Figure 4.5). The experiment was conducted three times with PBMCs from different buffy coat blood preparations; the results shown are representative of 3 individual patients.

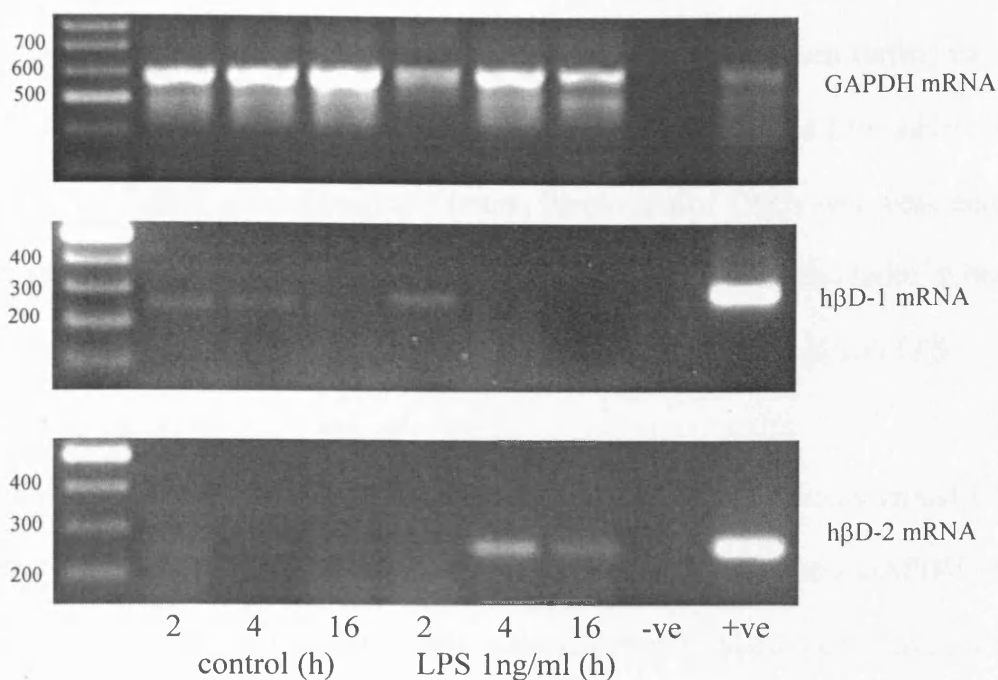


Figure 4.5 The expression of the housekeeping gene GAPDH, hβD-1 and hβD-2 mRNA in PBMCs from a healthy donor after incubation with 1ng/ml LPS. Results are representative of 3 separate experiments.

4.4.4 The effect of a CD14 blocking antibody MY4 on oral epithelial cell production of IL-8 protein and hβD-2 mRNA after stimulation with 1μg/ml *E. coli* LPS

H357 oral epithelial cells were stimulated in the presence of the anti-CD14 antibody MY4 to determine the effect on IL-8 protein and hβD-2 mRNA production. Cells were incubated as described in the methods section. Results for the production of IL-8 show that after 8 hours of incubation with 1μg/ml LPS, there was a significant 10-fold increase ($p < 0.0005$) in the amount of IL-8 released from oral epithelial cells. After an hour of pre-incubation with MY4, 1μg/ml LPS stimulated oral epithelial cells significantly ($p < 0.005$) to produce IL-8 but to a lesser degree. Only a 3-fold increase in IL-8 production compared to the control was detected. Analysis using Student's t-test showed that the MY4 significantly ($p < 0.0005$) blocked the secretion of IL-8 from oral epithelial cells compared to cells

stimulated with 1µg/ml LPS alone. Controls included pre-incubation with MY4 or the isotype control IgG2b, with no addition of LPS after an hour and then further incubation for 8 hours. Also, cells were pre-incubated with IgG2b and 1µg/ml LPS added after an hour and again further incubation for 8 hours. Incubation of OECs with both antibodies had minimal effect on IL-8 production. The control antibody IgG2b also failed to block the increased secretion of IL-8 from OECs when stimulated with 1µg/ml *E. coli* LPS.

After 8 hours incubation, mRNA was extracted from stimulated monolayers and RT-PCR performed to determine the mRNA levels of the housekeeping gene GAPDH and the antimicrobial peptide hβD-2. Agarose gels were analysed by densitometry using Phoretix 1D software, this software measures and compares the pixel intensity of bands like those shown in Figure 4.7. For the purposes of these experiments, bands were measured for GAPDH and hβD-2 from each sample and compared; hβD-2 is represented as percentage of the respective GAPDH and results expressed as arbitrary units. Results showed that all samples had similar levels of GAPDH mRNA, showing constitutive expression, which was not upregulated by 1µg/ml LPS, MY4 or IgG2b.

The level of hβD-2 mRNA was increased after 8 hours exposure to 1µg/ml LPS compared to the control, which is consistent with results shown previously in Figure 4.4. As seen with IL-8 protein production, after pre-incubation with MY4 and subsequent incubation with 1µg/ml LPS, expression of mRNA for hβD-2 in oral epithelial cells is lower than that seen after stimulation with 1µg/ml LPS alone. Densitometry results, as seen in Figure 4.8, show that whilst pre-incubation with MY4 and subsequent incubation with 1µg/ml LPS does still show a slight induction of hβD-2 mRNA, there is a significant reduction in the expression of hβD-2 mRNA compared to stimulation with 1µg/ml LPS alone. Pre-incubation with the control antibody IgG2b and subsequent incubation with 1µg/ml LPS

demonstrated that there was still a two-fold increase in production of h β D-2 mRNA. This shows that 1 μ g/ml LPS increases the expression of h β D-2 mRNA even after pre-incubation with IgG2b, and therefore the decrease in h β D-2 mRNA production can be said to be due to the blocking effect of MY4 on CD14. Both antibodies had minimal effect on h β D-2 mRNA expression in OECs. The experiment was repeated; results shown are representative of repeated experiments. Repeat experiments showed similar trends; however densitometry was only carried out on one of the repeat experiments and may not therefore reflect the true reproducibility of these experiments.

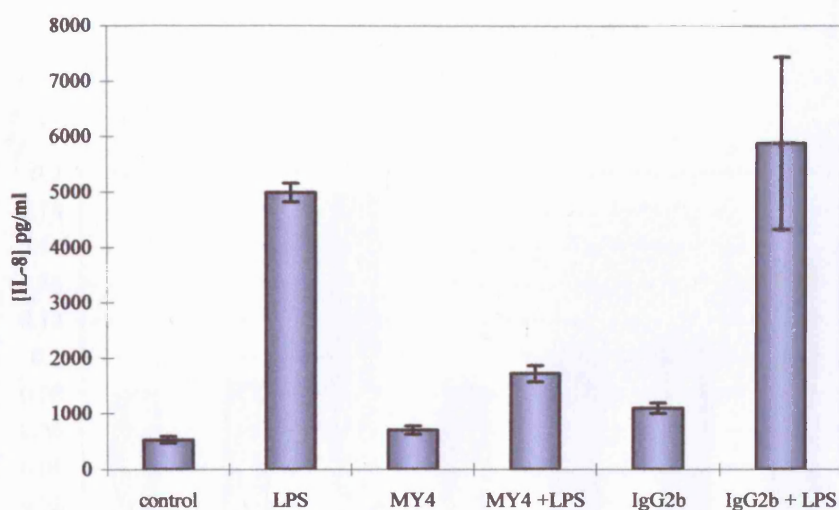


Figure 4.6 Effect of anti-CD14 MAb on IL-8 secretion by oral epithelial cells in response to bacterial LPS. IL-8 levels are expressed as the mean pg/ml \pm standard deviation of triplicate cultures. The results are representative of two different experiments.

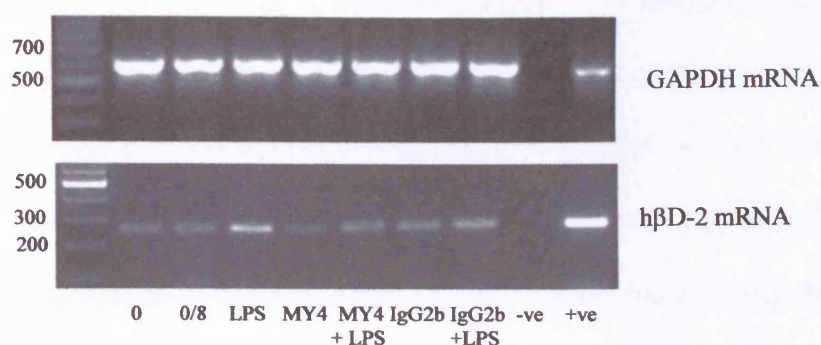


Figure 4.7 The effect of anti-CD14 MAb on the production of mRNA for hβD-2 and the housekeeping gene GAPDH in the oral epithelial cells

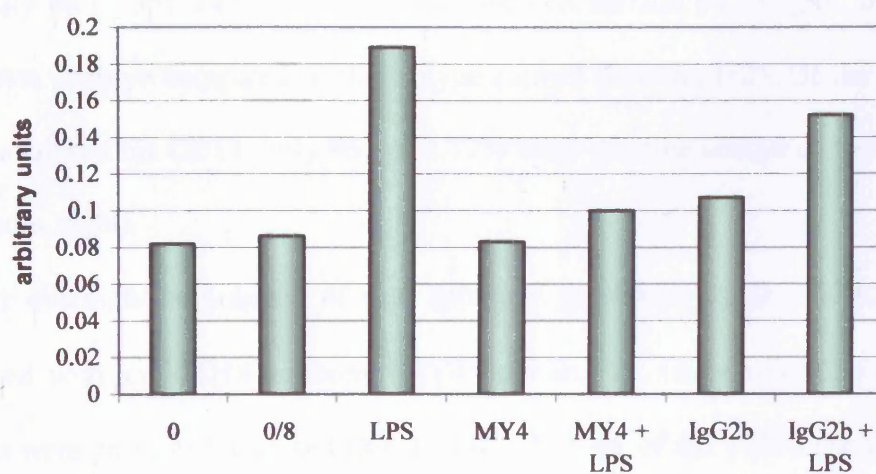


Figure 4.8 Densitometric analysis of bands for gapdh and hβD-2 PCR products as seen in figure 4.7. hβD-2 is expressed as a percentage of gapdh for each sample and shown as arbitrary units.

4.4.5 FACS analysis of oral epithelial cells for CD14

Since CD14 plays a role in the production of IL-8 and defensins in epithelial cells, FACS analysis was used to determine the presence of CD14 on the cell surface of the oral epithelial cell line H357. These cells constitutively express β -1 integrin (Sugiyama *et al.*, 1993) and this was used as a positive control. PBMCs were isolated and the presence of CD14 on the monocyte population was determined as a positive control. Monolayers of H357 oral epithelial cells and monocytes were also incubated with 1 μ g/ml LPS overnight to assess possible upregulation of cell surface CD14. Results obtained with the FACScan cytometer, in conjunction with CellQuest data acquisition, were analysed using WinMDI software, as previously described (see Chapter 2, section 2.9.1.2). Figure 4.9 shows the staining of H357 oral epithelial cells with anti-CD14 antibody (MY4) and anti- β 1 integrin (CD29). Of the 14534 oral epithelial cells gated and analysed in Figure 4.9, as seen in the density plot (top), 14532 were positive for cell surface β 1 integrin, this corresponds to 95.04% positive compared to the isotype control (bottom, left). Of the 13637 cells gated and analysed for CD14, only 961 or 1.77% were positive compared to the isotype control (bottom, right).

After overnight stimulation of oral epithelial cells with 1 μ g/ml LPS, cells were again stained with anti-CD14 antibody (MY4) and anti- β 1 integrin (CD29) (see Figure 4.10). Cells were gated and analysed (R1, Figure 4.10 top), of the 13072 gated cells 13071 were fluorescent positive for β 1 integrin, which equates to 95% (Figure 4.10, bottom, left). After staining with MY4 of the 13344 cells analysed, 685 cells were positive for CD14, representing 1.04% positive cells (Figure 4.10, bottom, right).

PBMCs isolated from human blood were stained with MY4, the anti-CD14 antibody, as a positive control (see Figure 4.11). From the density, plot the monocyte population were gated and analysed for anti-CD14 (Figure 4.11, left (R1)). Of the 15482 cells counted, 13968 were fluorescent-positive for CD14 on their cell surface, which equates to 90.2% of

gated cells. PBMCs were also incubated overnight with LPS at the lower concentration of 1ng/ml and then stained with 60bca to determine the presence of CD14 on their cell surface. Of the 18294 cells gated (Figure 4.12, left (R1)) 17094 were fluorescence positive for CD14 compared to the isotype control demonstrating that 93.4% of cells analysed were positive (Figure 4.12, right). This experiment was carried out three times; results shown are representative of all three experiments.

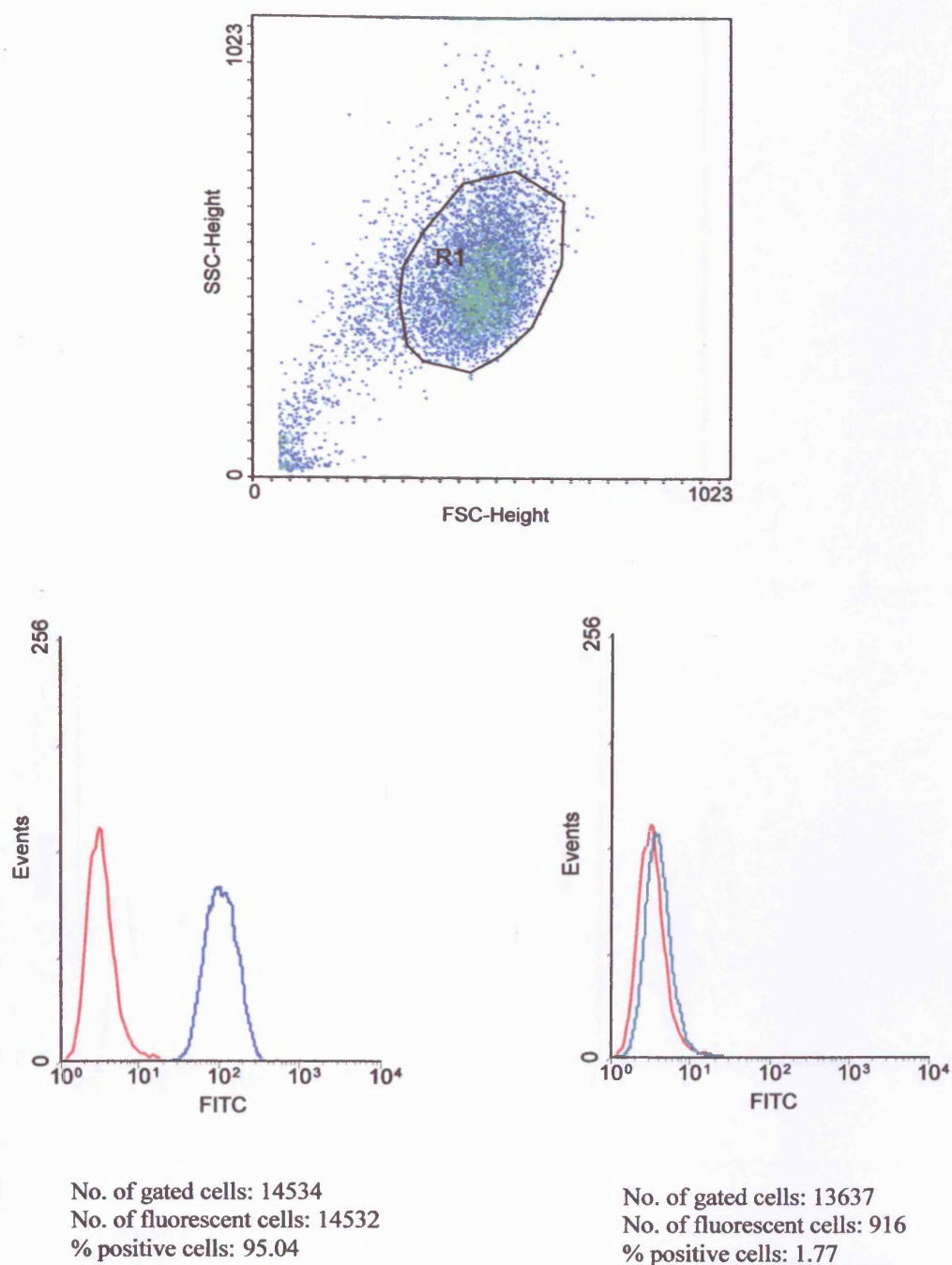


Figure 4.9 Staining of H357 oral epithelial cells with anti-CD14 antibody and anti-β1 integrin (CD29). Epithelial cells were identified and gated (R1) according to their forward and side light scattering properties, as seen in the density plot (top). Cells within this gate were then analysed for anti-CD14 and anti-β1 integrin. Cells were stained with anti-CD14 mAb, MY4 (green peak), anti-β1 integrin (blue peak) or with the isotype control IgG2b (red peak) as shown in the lower graphs. Fluorescence emitted by the cells is plotted along the x-axis and the number of cells counted are plotted along the y-axis. Statistical analysis performed using WinMDI is displayed under the histograms.

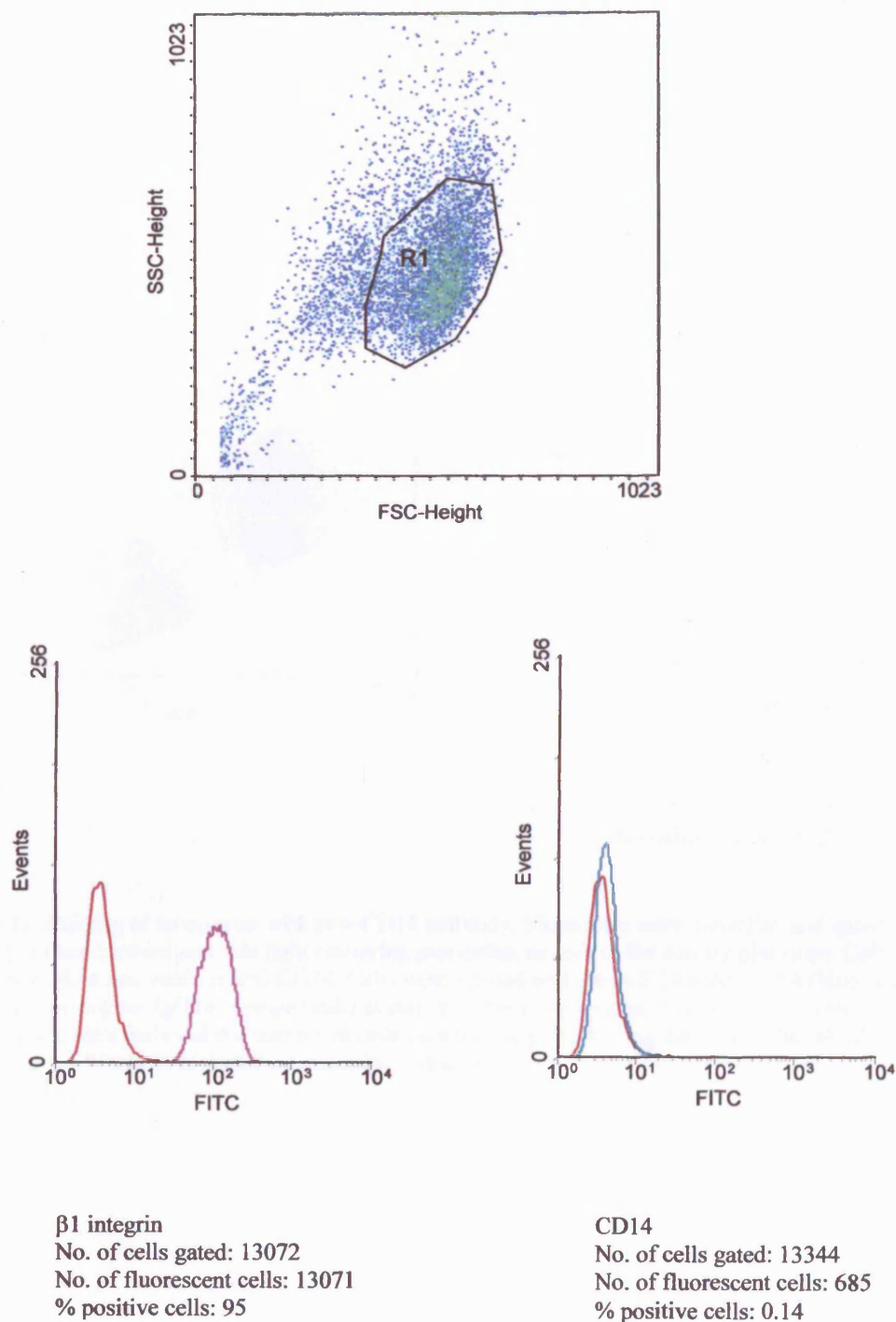


Figure 4.10 Staining of H357 oral epithelial cells with anti-CD14 antibody and anti-β1 integrin (CD29) after overnight stimulation with 1 μg/ml LPS. Epithelial cells were identified and gated (R1) according to their forward and side light scattering properties, as seen in the density plot (top). Cells within this gate were then analysed for anti-CD14 and anti-β1 integrin. Cells were stained with anti-CD14 mAb, MY4 (green peak), anti-β1 integrin (purple peak) or with the isotype control IgG2b (red peak) as shown in the lower graphs. Fluorescence emitted by the cells is plotted along the x-axis and the number of cells counted are plotted along the y-axis. Statistical analysis performed using WinMDI is displayed under the histograms.

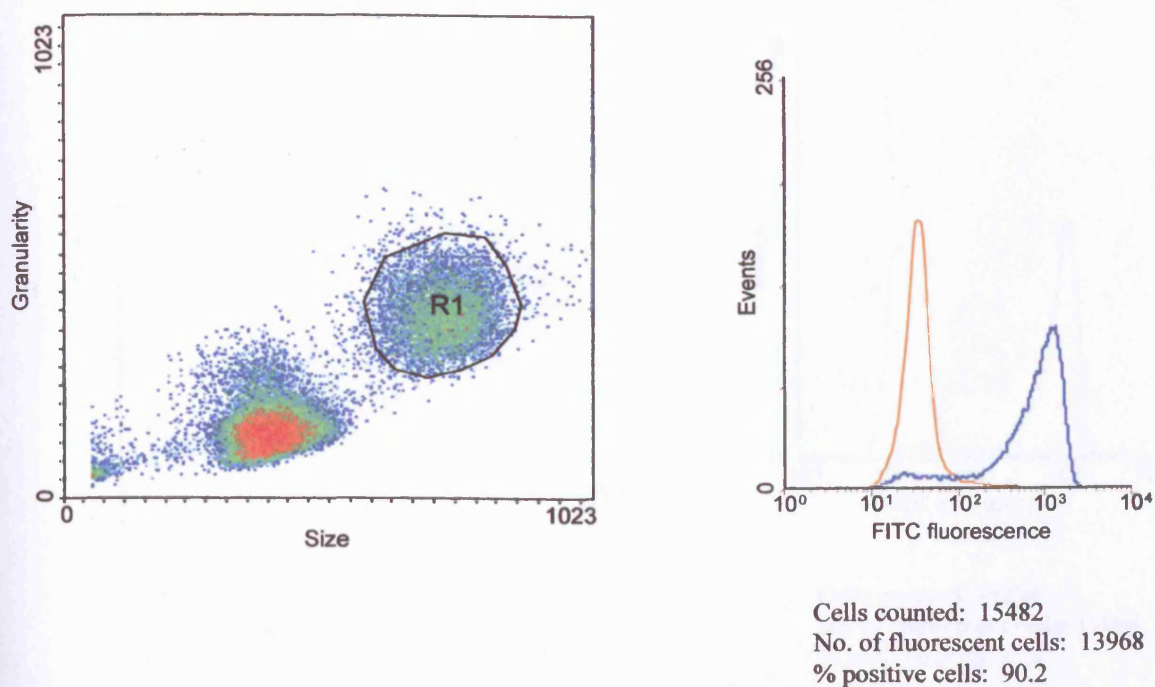


Figure 4.11 Staining of monocytes with anti-CD14 antibody. Monocytes were identified and gated (R1) according to their forward and side light scattering properties, as seen in the density plot (top). Cells within this gate were then analysed for anti-CD14. Cells were stained with anti-CD14 mAb, MY4 (blue peak), or with the isotype control IgG2b (orange peak) as shown in the lower graphs. Fluorescence emitted by the cells is plotted along the x-axis and the number of cells counted are plotted along the y-axis. Statistical analysis performed using WinMDI is displayed under the histograms.

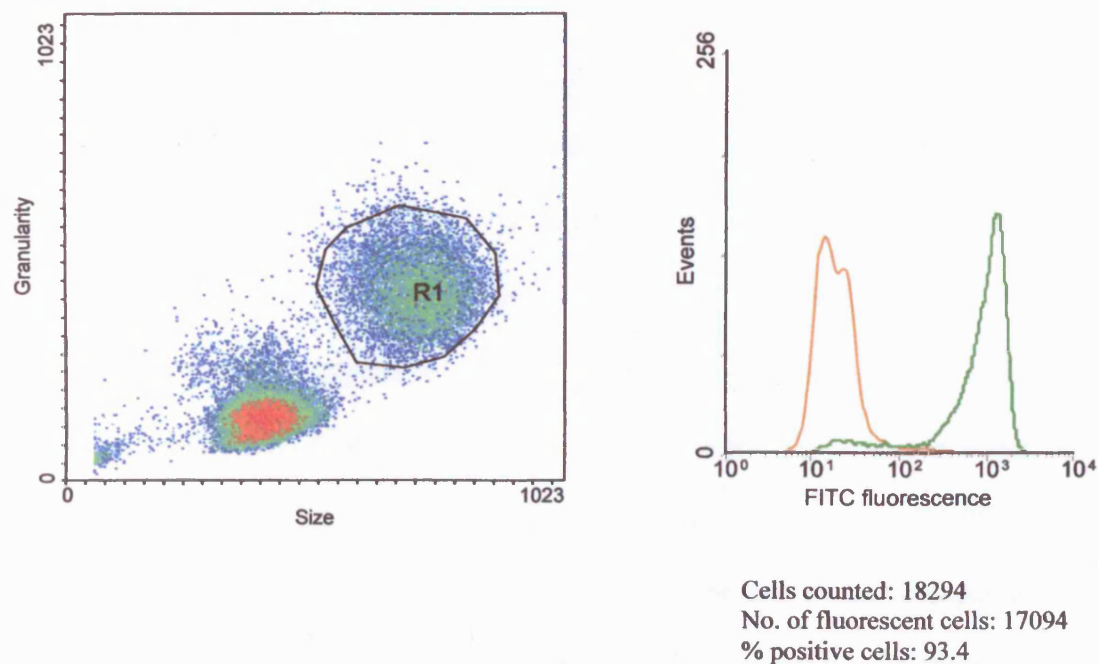


Figure 4.12 Staining of monocytes with anti-CD14 antibody after overnight incubation with 1 ng/ml LPS. Monocytes were identified and gated (R1) according to their forward and side light scattering properties, as seen in the density plot (top). Cells within this gate were then analysed for anti-CD14. Cells were stained with anti-CD14 mAb, MY4 (green peak), or with the isotype control IgG2b (orange peak) as shown in the lower graphs. Fluorescence emitted by the cells is plotted along the x-axis and the number of cells counted are plotted along the y-axis. Statistical analysis performed using WinMDI is displayed under the histograms.

4.5 Discussion

A few limited reports have been published about the responses of human gingival keratinocytes to *E. coli* LPS in relation to the production of h β D-1 and h β D-2 mRNA (Mathews *et al.*, 1999, Krisanaprakornkit *et al.*, 2000). This chapter demonstrates a more detailed investigation into the presence of h β D-2 mRNA in an oral epithelial squamous cell carcinoma (SCC) cell line, and the effect of *E. coli* LPS on these cells. The oral epithelial cell line H357, which was derived from a SCC of the tongue, responded to *E. coli* LPS by increased production of IL-8 protein and h β D-2 mRNA. Messenger RNA for h β D-2 was found to be upregulated in a time and dose-dependent fashion.

The results of studies reported in this chapter show that oral epithelial cells are also induced by *E. coli* LPS to produce IL-8 protein in a time- and dose-dependent fashion. However, RT-PCR analysis of oral epithelial cells incubated with *E. coli* LPS showed no upregulation of IL-8 mRNA at the doses and time points tested, compared to their corresponding controls. The oral epithelial cell line H357 used in this study produces a high level of IL-8 without any stimulation, increasing in a time-dependent fashion.

Other studies conducted at the same time as those presented above have shown that treatment of primary gingival epithelial cells for 24h with 1 μ g/ml *E. coli* LPS induced a ~5-fold increase in h β D-2 mRNA expression (Mathews *et al.*, 1999). Similarly, primary gingival epithelial cells express h β D-1 and h β D-2 mRNA, and h β D-2 expression was upregulated by stimulation with *E. coli* LPS at a lower concentration of 100ng/ml (Abiko *et al.*, 1999). However, Krisanaprakornkit *et al.*, 2000 found only slight induction of mRNA for h β D-2 in HGE stimulated with the maximum dose (1 μ g/ml) of *E. coli* LPS for 24h.

Oral squamous cell carcinoma (OSCC) cell lines Ca-9 (gingiva SCC derived), SCC-9, and HSC-4 (tongue SCC derived) express h β D-1, whilst Ca-9, SCC-9, HSC-4, SAS (tongue

SCC derived) and KB cell lines express h β D-2. In one of these cell lines, SCC-9, h β D-2 mRNA is upregulated by *E. coli* LPS and TNF- α (Abiko *et al.*, 1999).

The results presented herein are consistent with those of Abiko *et al.*, 1999, however in this study it was observed that oral epithelial cells were stimulated by *E. coli* LPS to produce h β D-2 mRNA after only 2 hours. Abiko *et al.*, 1999 stimulated their cell lines for 24 hours before analysis of h β D-2 mRNA.

All of the studies described have used cell lines from different oral sites and in those studies where primary gingival epithelial cells have been utilised, the explants from which the cells were derived have come from different individuals. This suggests that the expression of h β D-2 in oral epithelial cells differs from site to site and between individuals.

The present study used the H357 OSCC cell line derived from the tongue of a 73 year old male. It may be that these tongue-derived cells, as they are constantly exposed to oral bacteria, may normally express high levels of h β D-2 so that the oral mucosa maintains homeostasis and balance in the bacteria present. Gingival epithelium in the oral mucosa, unlike the tongue, is subjected to daily oral hygiene practises, which aids in the removal of excess oral bacteria, which may cause periodontal problems.

The mammalian tongue contains a dense epithelium that is constantly colonised by the microbiota of the mouth, which includes bacteria, fungi and viruses. Although abrasions to the surface of the tongue occur often, invasive infections in the normal host are rare, remain localised, and heal rapidly. Invasive infections of the tongue would interfere with the processes of chewing, swallowing, taste and speech (Nally, 1991). Schonwetter *et al.*, 1995 posed the question, why is this exposed surface free of continuous infection? To answer the question they determined whether the epithelium of the tongue produces antibiotic agents capable of providing a broad-spectrum chemical shield. They found an epithelial β -defensin, which they termed lingual antimicrobial peptide (LAP), isolated

from bovine tongue. LAP showed a broad spectrum of antibacterial and antifungal activities, also LAP mRNA abundance was markedly increased in the epithelium surrounding naturally-occurring tongue lesions (Schonwetter *et al.*, 1995). It could be that h β D-2 is the human equivalent of bovine LAP, and is expressed at a high level in human tongue epithelial tissue. h β D-2 shares 46.3% amino acid sequence homology to bovine LAP, compared to 28.8% homology with h β D-1. The expression of h β D-2 mRNA and protein in normal tongue epithelial biopsies has been reported but has not been studied extensively (Mathews *et al.*, 1999, Dunsche *et al.*, 2001 and Abiko *et al.*, 2001).

The oral epithelial cell line H357 would therefore provide an ideal model to investigate the effects PAMPs from oral bacteria, and the oral bacteria themselves, have on the production of h β D-2 mRNA.

Since *E. coli* LPS has been found to act through CD14 on human keratinocytes, the role of CD14 in the upregulation of IL-8 and h β D-2 in the oral epithelial cell line H357 was determined. Significant blocking of IL-8 protein upregulation by *E. coli* LPS after oral epithelial cells were pre-incubated with the anti-CD14 antibody MY4 was observed. This suggests that in the oral epithelial cell system used *E. coli* LPS upregulates IL-8 in a CD14-dependent manner. Further studies showed that incubation of oral epithelial cells with MY4 prior to stimulation with *E. coli* LPS blocked the upregulation of h β D-2 mRNA normally observed in these oral epithelial cells after stimulation with *E. coli* LPS. This suggests a role for CD14 in the upregulation of h β D-2 in H357 oral epithelial cells. However, subsequent flow cytometric analysis revealed the absence/low expression of membrane CD14 in these oral epithelial cells. Using RT-PCR the presence of CD14 mRNA was detected in the H357 oral epithelial cell line, this observed expression was unaffected by stimulation of the cell line with *E. coli* LPS. As the pre-incubation of oral epithelial cells with MY4 did not completely knockout the IL-8 upregulation demonstrated

after stimulation with *E. coli* LPS, another receptor may be involved in IL-8 protein and h β D-2 mRNA upregulation in these cells.

Studies by Uehara *et al.*, 2001 have shown that oral epithelial cells (primary and the KB cell line) are devoid of membrane CD14, as determined by flow cytometry, and lack CD14 mRNA expression, as determined by RT-PCR. So similarly, the results of this study also showed the lack/or low expression of membrane CD14 on the oral epithelial cells used, however, the expression of CD14 mRNA in oral epithelial cell has been demonstrated. As mentioned previously, the H357 oral epithelial cell line production of IL-8 protein and h β D-2 mRNA in response to *E. coli* LPS exposure is blocked after cells are pre-incubated with the anti-CD14 antibody, MY4. The oral epithelial cell line used in this study may utilise soluble CD14 (sCD14) contained in the medium in which the cells are incubated, the anti-CD14 antibody MY4 could possibly bind to sCD14 in the cell culture medium rendering it unable to bind to the epithelial cells. In CD14⁺ cells such as endothelial cells, granulocytes and lymphocytes, sCD14 found in serum is thought to functionally replace membrane-bound CD14 (Schletter *et al.*, 1995, Ulevitch and Tobias 1995). Binding of LPS to CD14 requires the serum factor LPS-binding protein (LBP), which delivers LPS to CD14 expressing monocytes/macrophages (Schletter *et al.*, 1995, Ulevitch and Tobias 1995). Cell culture serum may also provide a source of LBP, which aids CD14 binding to oral epithelial cells.

Other recent studies have demonstrated that the CD14-specific antibody, MY4, inhibited LPS-induced h β D-2 mRNA expression in hTBE cells, suggesting a critical role for CD14 in the mechanism by which airway epithelial cells recognise and respond to bacterial products. The study also demonstrated the presence of CD14 mRNA and cell surface protein in hTBE cells (Becker *et al.*, 2000).

It is possible that the surface level of CD14 in the H357 oral epithelial cell line is at such a low level, so as to be just within the level of detection in the flow cytometry analysis,

further studies would be needed to determine the levels of CD14 on these oral epithelial cells. Like IL-8 protein, the upregulation of h β D-2 mRNA in the oral epithelial cells may require the presence of sCD14 in the cell culture serum, which is blocked after incubation with anti-CD14, MY4. Either way the presence of CD14 is required in oral epithelial cell H357 for response to *E. coli* LPS with the upregulation of h β D-2.

Because CD14 is a GPI-anchored protein that lacks an intracytoplasmic signalling domain, other intra-cellular molecules are required for cellular responses to LPS. Studies have demonstrated that LPS may not only bind to CD14 but also TLR proteins either in conjunction with a CD14 complex or independently (Yang *et al.*, 1998). Multiple types of TLR have been described on mammalian cells and so it has been proposed that the innate immune system may use different combinations of TLRs to recognise different groups of microbial pathogens.

Becker *et al.*, (2000) found that the LPS induction of h β D-2 in hTBE cells requires CD14, and suggest that this may complex with a TLR to ultimately activate NF- κ B. Birchler *et al.*, (2001) showed that exposure of human cells to synthetic bacterial lipoprotein elicits production of the antimicrobial peptide β -defensin 2 through TLR2.

H357 oral epithelial cells express mRNA for TLR2 and TLR4, although TLR4 mRNA is less abundant than TLR2. This is consistent with results published by Becker *et al.*, (2000) who found that human TLR4 expression is detectable by northern blot but is much less abundant than TLR2 in hTBE cells. They also found that neither TLR2 nor TLR4 mRNA expression are regulated by *E. coli* LPS. Results presented in this chapter also show no upregulation of TLR2 and TLR4 mRNA in oral epithelial cells after stimulation with *E. coli* LPS. Whilst this study has shown the presence of TLRs mRNA in the oral epithelial cell line H357, further studies would need to be carried out to determine their role in the

induction of h β D-2 mRNA, indeed if there is one. This would possibly require the development of tools such as neutralising antibodies analogous to MY4.

This study reports, for the first time, that human monocytes express mRNA for h β D-1 and h β D-2 and that they are regulated by *E. coli* LPS. We found that PBMCs isolated from healthy blood donors were shown to express mRNA for h β D-1 at 2 and 4 hours but not after 16h incubation. This expression was unaffected after PBMCs were stimulated with 1ng/ml *E. coli* LPS. In contrast, whilst h β D-2 could be seen at very low levels in control samples at all time points, expression of h β D-2 mRNA was clearly increased after stimulation with 1ng/ml *E. coli* LPS at 4 and 16 hours.

The results concerning h β D-1 mRNA expression in monocytes are consistent with results shown for h β D-1 mRNA in oral keratinocytes where it is produced constitutively and is not up-regulated by pro-inflammatory stimuli (Zhao *et al.*, 1996, McCray *et al.*, 1997). In this study, increased expression of h β D-2 in human monocytes after exposure to *E. coli* LPS was observed, these results are also consistent with results found in other cell types including keratinocytes (Harder *et al.*, 1997) and airway epithelial cells (Singh *et al.*, 1998, Harder *et al.*, 2000). With respect to h β D-1 mRNA, expression was found to disappear after monocytes had been in culture for 16 hours.

It may be that h β D-1 is produced constitutively as a constant defence against invading microorganisms. h β D-2 may be upregulated when an infection becomes more severe, h β D-2 is also well placed to be sequentially involved in orchestrating an immune response since it has been found that β -defensins may attract immature dendritic cells, naïve T cells and memory T cells, whilst h β D-1 also is chemotactic for these cells, it is to a much lesser extent (Yang., 1999a). If the same mechanism functions *in vivo*, the release of these defensins from injured epithelial cells, and also monocytes at sites of infection, would

recruit dendritic cells and memory T cells to infected tissues, thereby promoting the development of adaptive (antibody and T-cell mediated) immunity.

Chapter 5

The stimulatory effect of soluble bacterial factors on oral epithelial cells

Chapter 5

The stimulatory effect of soluble bacterial and other factors on oral epithelial cells

5.1 Introduction

It is essential for the innate immune system to immediately recognise the presence of disease-inducing bacteria (and other microbial pathogens). Janeway and collaborators have, over the last decade, argued that the innate immune system recognises key molecular structures of pathogens (Janeway, 1992). These molecules are essential for bacterial survival and therefore unlikely to evolve their structures, as any mutation would be lethal for the microorganism. Such molecules would include structural components such as LPS, peptidoglycan and LTA. These molecules are present in all bacteria and Janeway has proposed that the key feature of the innate immune recognition system is that it recognises, not individual molecules, but patterns of molecules, for which the term pathogen-associated molecular patterns (PAMPs) has been coined. They have also predicted that host organisms will have developed a group of receptors that recognise these PAMPs and these have been referred to as pattern recognition receptors (PRRs).

Most workers investigating the chronic inflammatory periodontal diseases have concentrated on LPS. However, the Cellular Microbiology Research Group (CMRG) at the Eastman Dental Institute have shown that in studies on bone breakdown induced by oral bacteria LPS is less active than surface-associated components (Wilson *et al.*, 1985, Medhji *et al.*, 1994, Kirby *et al.*, 1995). The CMRG has demonstrated that the *E. coli* cpn 60 (GroEL), but not that of *Mycobacterium tuberculosis* and *Mycobacterium leprae*, is a potent stimulator of murine bone resorption *in vitro* (Kirby *et al.*, 1995) and that such resorption can be inhibited by neutralising the activity of IL-1 with IL-1ra (Nair *et al.*, 1997). Also, the CMRG have demonstrated that the surface-associated material from *A. actinomycetemcomitans*, in contrast to the LPS from this bacterium, is a potent inducer

of cytokine synthesis by human PBMCs, the myelomonocytic cell line Mono-Mac-6 and human gingival fibroblasts (Reddi *et al.*, 1996).

Mucosal surfaces are the most frequent route of entry of microbial pathogens into the host. Epithelial cells which line the mucosal surface are an important mechanical barrier that separates the host from the external environment. Studies over recent years have led to the concept of epithelial cells as an integral component of a communications network which involves interactions between epithelial cells, microbes and host immune and inflammatory cells. Whilst studies have determined the action of bacterial surface components on cells of the immune system, little has been published on the effect of these components on the epithelial cell.

5.2 Aims

The purpose of this study was to determine whether the bacterial components *A. actinomycetemcomitans* SAM, *E. coli* GroEL, *P. gingivalis* LPS and *S. sanguis* LTA could stimulate the production of immune factors (human beta-defensin 2, IL-8 and SLPI) by the oral epithelial cell line H357 in vitro. The presence of mRNA for TLR2 and TLR4, which may be possible receptors for these molecules, was also investigated. The effect these bacterial surface components may have on the expression of TLRs in oral epithelial cells was also determined.

5.3 Materials and methods

Stimulatory factors

- *E. coli* LPS, 055:B5, Becton Dickinson, Oxford, UK
- *S. sanguis* LTA, Sigma, Poole, UK
- *A. actinomycetemcomitans* SAM, provided by Ms Lindsay Sharp. SAM was extracted as described in Wilson *et al.*, 1985 from *A. actinomycetemcomitans* strain NCTC 9710
- *E. coli* GroEL, provided by Dr. Sahar Khan. The protein was prepared as described in Tabona *et al.*, 1998
- Polymyxin B (PB), Sigma, Poole, UK. PB is an LPS-binding and inactivating antibiotic.
- Phorbol 12-myristate 13-acetate (PMA), Sigma, Poole UK

The cell line H357 was used for the experiments described in this chapter. Cells were maintained as in Chapter 2, section 2.2.1. For the purposes of these experiments, cells were prepared and treated as follows.

To T75 flasks confluent with H357 cells, 1ml of trypsin was added and incubated at 37°C for 10 minutes until cells had detached from the surface. After addition of fresh medium, cells were centrifuged at 1500rpm for 5 minutes and resuspended in 10ml of fresh medium. Cells were counted, diluted accordingly with medium and seeded into 6-well tissue culture plates to achieve a density of 125,000 cells per well. Tissue culture plates were then incubated at 37°C/5% CO₂ for approximately 40 hours until cells were 80% confluent. Monolayers were then washed with PBS and 3ml of fresh medium added. The appropriate stimulant was added, diluted in PBS, and cells incubated for the times indicated in the figures. Control cells were incubated in KGM alone. After incubation, 1ml of medium was removed, placed in a 1.5ml eppendorf and frozen at -70°C until an IL-8 ELISA, as

described in Chapter 2, section 2.3, was carried out. The remaining medium was removed from the cells, which were then washed with PBS. Monolayers were then lysed by addition of 600µl Solution D and the plates stored at -70°C until RNA was extracted and analysis by RT-PCR as detailed in Chapter 2, section 2.4-2.6. The number of PCR cycles for GAPDH was 26 and 35 for hβD-2, IL-8, SLPI, TLR2 and TLR4, primers and programs were as described in Chapter 2, table 2. 20µl of PCR product was loaded and run on a 2% agarose gel as described in Chapter 2 section 2.7.

5.4 Results

Maximal IL-8 protein was found to be secreted from oral epithelial cells after 8 hours incubation with 1µg/ml LPS as previously determined in Chapter 4, section 4.3.2. Therefore, various bacterial and other stimulatory factors were incubated for the same time period to assess their effects in this chapter.

In all experiments, *E. coli* LPS at a concentration of 1µg/ml stimulated an increase in the production of IL-8 protein from oral epithelial cells. Upregulation of IL-8 protein production from oral epithelial cells by *E. coli* LPS (1µg/ml) was blocked by pre-incubation of the cells with Polymyxin B see sections 5.4.1.1 and 5.4.2.1. In all experiments, cell monolayers showed constitutive expression of GAPDH mRNA. All oral epithelial cells stimulated with *E. coli* LPS at a concentration of 1µg/ml showed an increase in the expression of hβD-2 mRNA. The increased expression of hβD-2 mRNA, stimulated by *E. coli* LPS (1µg/ml), was blocked when oral epithelial cells were pre-incubated with Polymyxin B (Figures 4.2 and 4.4). Some agarose gels were analysed by densitometry using Phoretix 1D software, this software measures and compares the pixel intensity of bands. For the purposes of these experiments, bands were measured for GAPDH and target mRNA from certain samples and compared, the target mRNA is represented as a percentage of the respective GAPDH.

5.4.1 The effect of *A. actinomycetemcomitans* SAM on oral epithelial cells

5.4.1.1 IL-8 protein

Only the higher concentration of 10 µg/ml *A. actinomycetemcomitans* SAM stimulated significantly increased secretion of IL-8 protein from oral epithelial cells ($p < 0.05$). However, after pre-incubation with Polymyxin B, the stimulatory effect of 10 µg/ml *A. actinomycetemcomitans* SAM was no longer seen, suggesting the stimulatory action observed was a result of either LPS contamination or another factor which is, like LPS, neutralised by Polymyxin B. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

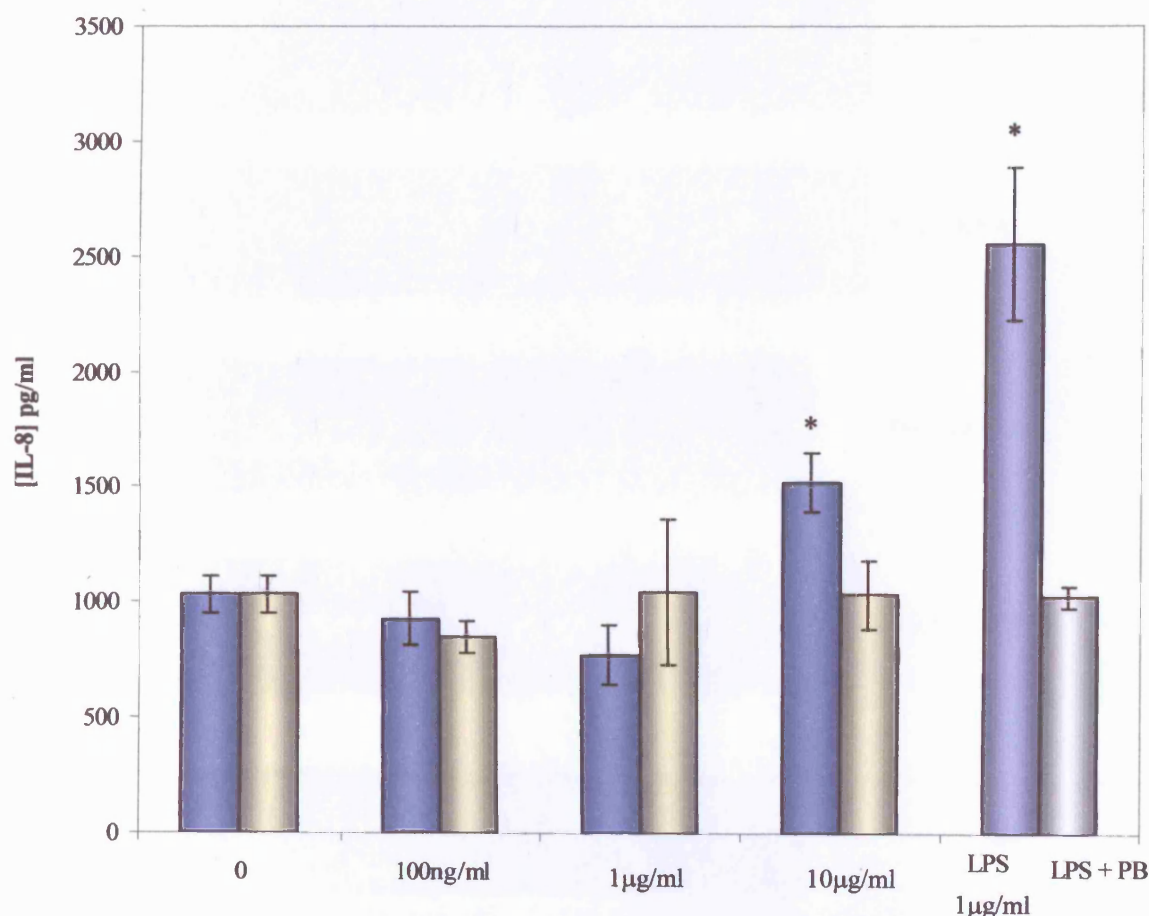


Figure 5.1 IL-8 production from oral epithelial cells following treatment with increasing concentrations of *A. actinomycetemcomitans* SAM for 8 h.
 ■ Aa SAM ■ Aa SAM and Polymyxin B. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures
 * $P < 0.05$ compared with control

5.4.1.2 RT-PCR

A. actinomycetemcomitans SAM had no effect on the expression of IL-8, SLPI, TLR2 and TLR4 mRNA. Results show that h β D-2 mRNA was upregulated in oral epithelial cells after treatment with all concentrations of *A. actinomycetemcomitans* SAM irrespective of pre-treatment with Polymyxin B. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

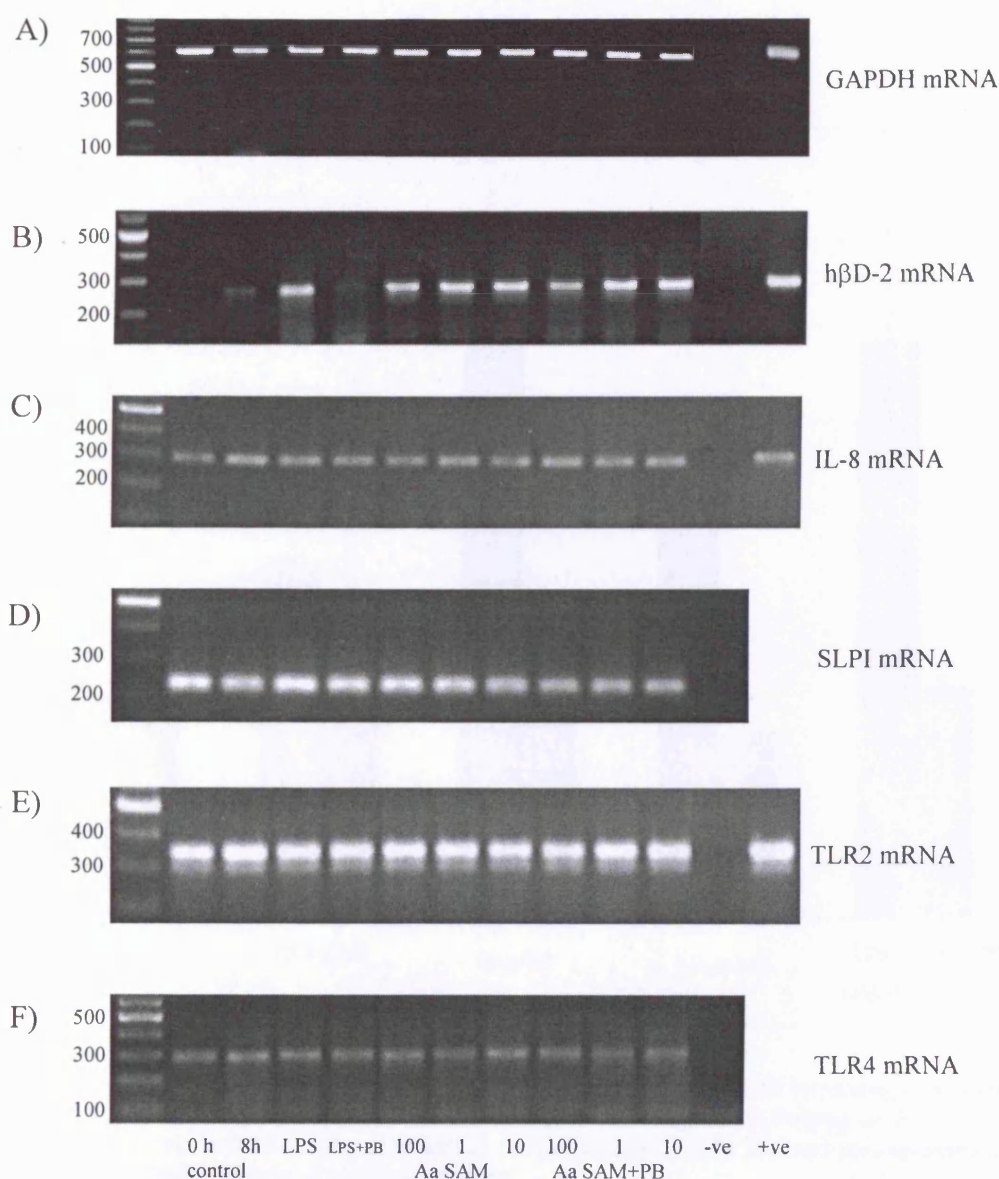


Figure 5.2 RT-PCR to show relative levels of mRNA obtained from oral epithelial cells treated with 100ng/ml, 1 μ g/ml and 10 μ g/ml *A. actinomycetemcomitans* SAM for 8h. Results show mRNA for a)the housekeeping gene GAPDH, b)h β D-2, c)IL-8, d)SLPI, e)TLR2 and f)TLR4

5.4.2 The effect of *E. coli* GroEL on oral epithelial cells

5.4.2.1 IL-8 protein

Incubation of oral epithelial cells with all concentrations of *E. coli* GroEL tested induced increased secretion of IL-8. However, pre-incubation of oral epithelial cell monolayers with Polymyxin B knocked out the stimulatory effect of *E. coli* GroEL. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

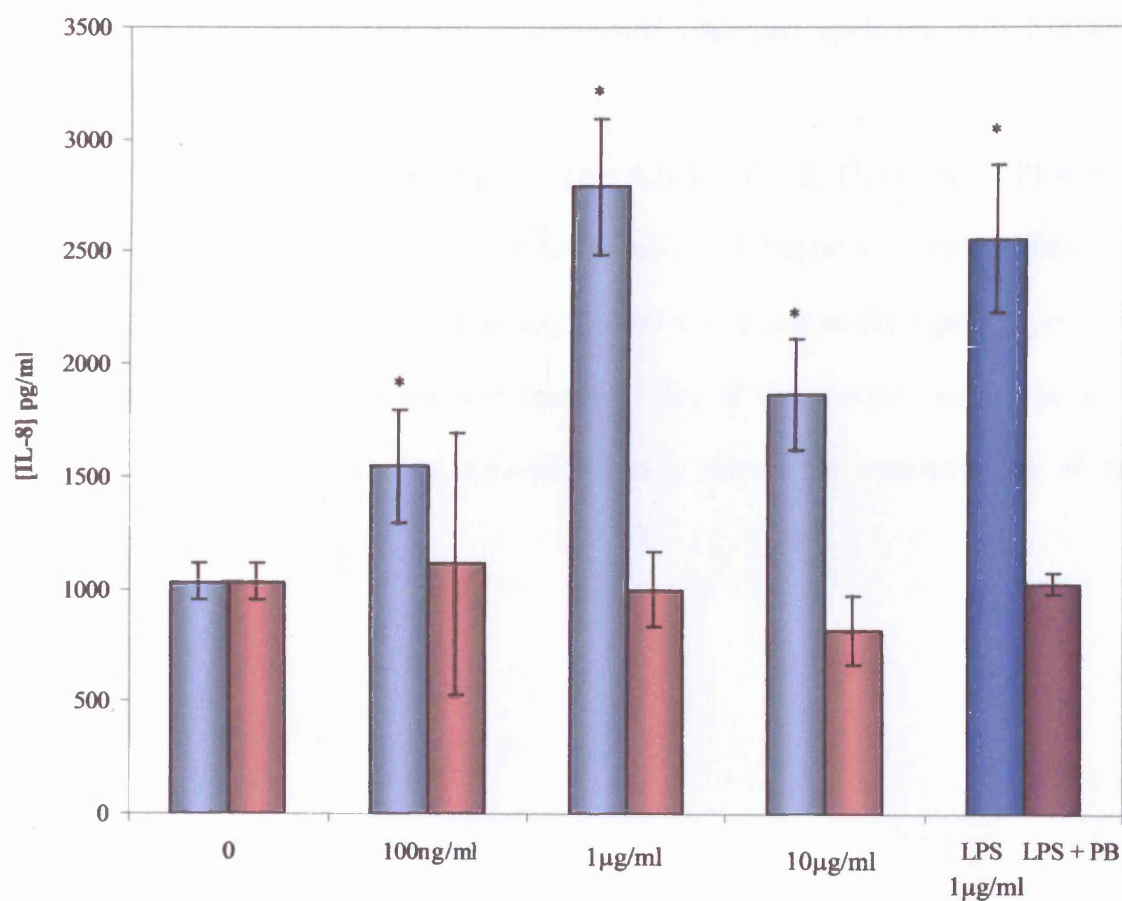


Figure 5.3 IL-8 production from oral epithelial cells following treatment with increasing concentrations of *E. coli* GroEL for 8 h. ■ *E. coli* GroEL ■ *E. coli* GroEL and Polymyxin B. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures
*, $P < 0.05$ versus control

5.4.2.2 Messenger RNA

Unfortunately, the mRNA pellet for cells pre-incubated with Polymyxin B and then stimulated with 100ng/ml *E. coli* GroEL was lost during processing of samples.

Incubation of oral epithelial cells with all concentrations of *E. coli* GroEL had no effect on the level of mRNA transcripts for TLR2. The amount of hβD-2 message detected in oral epithelial cells stimulated with all concentrations of *E. coli* GroEL for 8 hours was increased compared to the control and was unaffected by pre-incubation with Polymyxin B. Increased IL-8 mRNA was detected after incubation with 10μg/ml *E. coli* GroEL, however this increase was not as substantial after oral epithelial cells had been pre-incubated with Polymyxin B.

The results seen in the agarose gel (Figure 4.4) for TLR2, TLR4 and SLPI were further substantiated by gel image analysis using Phoretix 1D. Repeat experiments showed similar trends, however densitometry was only carried out on one of the repeat experiments and may not therefore reflect the true reproducibility of these experiments. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

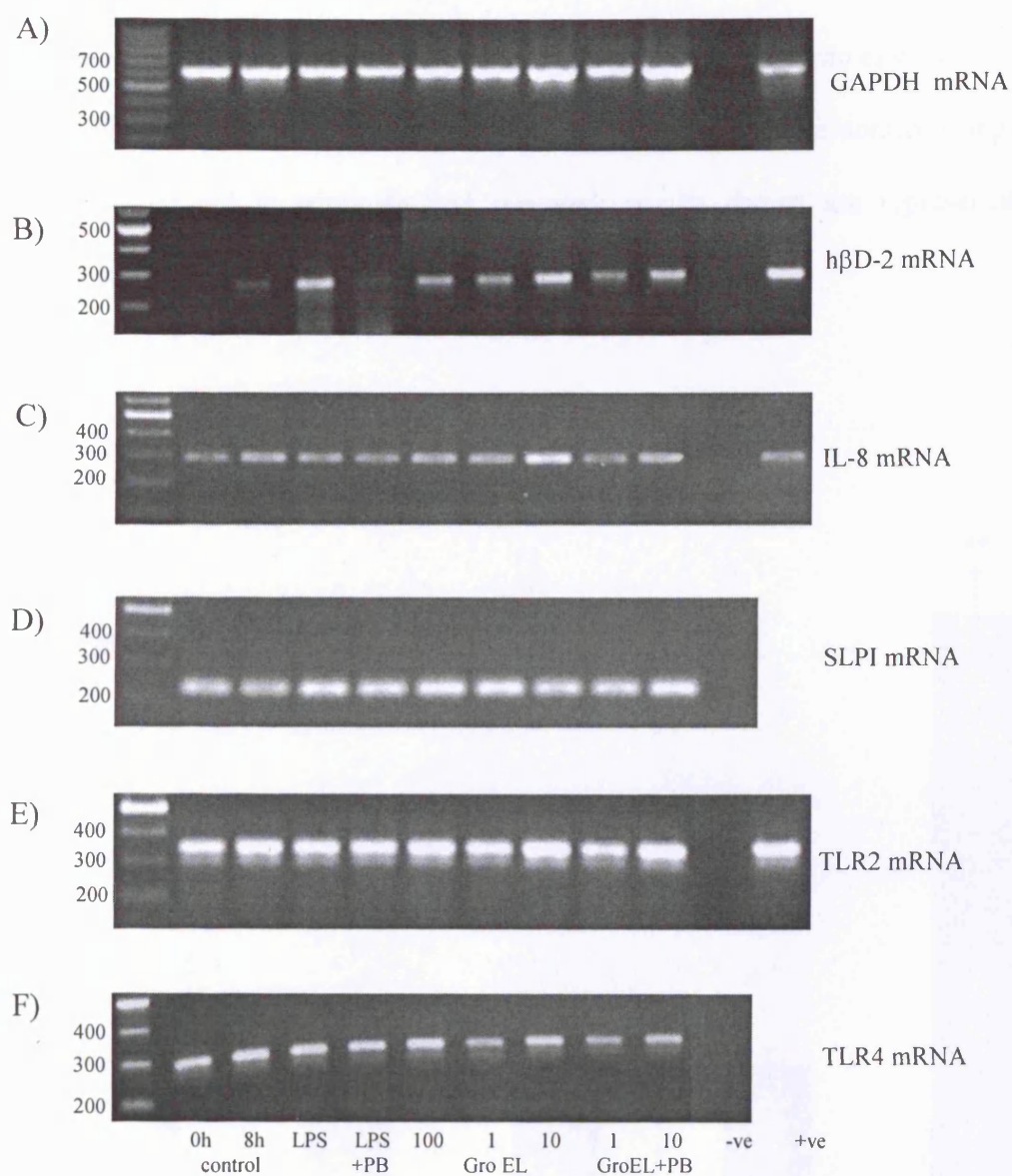


Figure 5.4 RT-PCR to show relative levels of mRNA obtained from oral epithelial cells treated with 100ng/ml, 1μg/ml and 10μg/ml *E. coli* GroEL for 8h. Results show mRNA for a) the housekeeping gene GAPDH, b) hβD-2, c) IL-8, d) SLPI, e) TLR2 and f) TLR4

5.4.3 The effect of *P. gingivalis* LPS on oral epithelial cells

5.4.3.1 IL-8 protein

Cell monolayers were incubated for 8 hours with 3 concentrations of *P. gingivalis* LPS, 100ng/ml, 1µg/ml and 10µg/ml. Measurement of IL-8 secreted from oral epithelial cells after 8 hours showed that all concentrations of *P. gingivalis* LPS had no effect on the levels of IL-8 protein detected in cell culture supernatant compared to the control sample. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

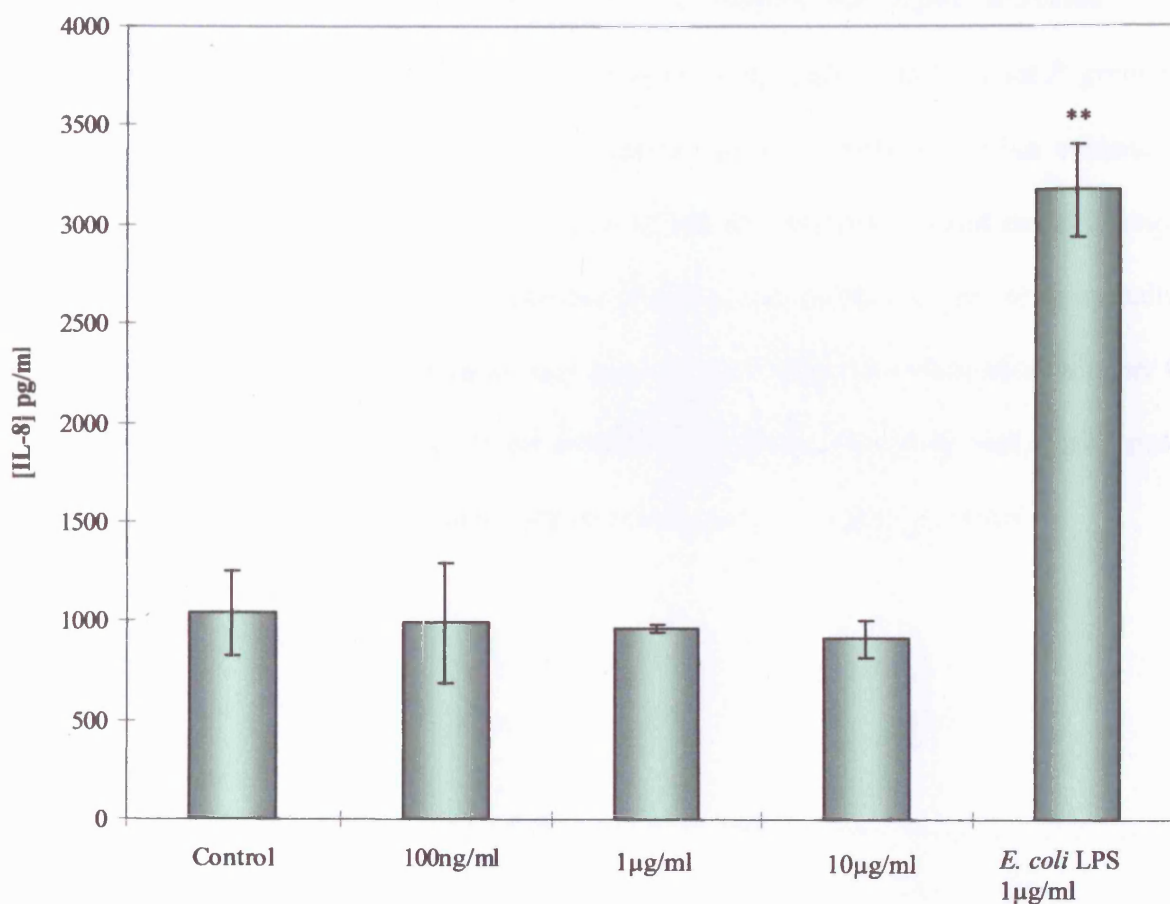


Figure 5.5 IL-8 production from oral epithelial cells following treatment with *P. gingivalis* LPS and 1µg/ml *E. coli* LPS for 8 hours. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures

** $p < 0.005$ versus control

5.4.3.2 Messenger RNA

Results are shown in Figure 4.7. All concentrations of *P. gingivalis* LPS had no effect on the IL-8 and TLR4 mRNA levels in oral epithelial cells. Gel images for hβD-2, SLPI, TLR2 and TLR4 were further analysed using Phoretix 1D to determine the difference in density of the bands between samples, each target mRNA was compared to the housekeeping gene GAPDH. Densitometry suggests a slight increase in the expression of hβD-2 mRNA in oral epithelial cells was seen after incubation with 100ng/ml and 10µg/ml *P. gingivalis* LPS. Densitometry analysis of SLPI mRNA also showed a slight increase in expression in oral epithelial cells after stimulation with all concentrations of *P. gingivalis* LPS. Messenger RNA for TLR2 was present in all samples, and slightly increased mRNA levels were shown in oral epithelial cells stimulated with 1µg/ml and 10µg/ml *P. gingivalis* LPS. The use of densitometry in this instant proved to provide no further evidence to whether an obvious difference in expression of mRNA between test and control samples existed. The use of PCR where the number of cycles was controlled for, whereby halting the reaction in the exponential phase may have given a better indication as to whether the stimulants tested had any effect on the mRNA investigated. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

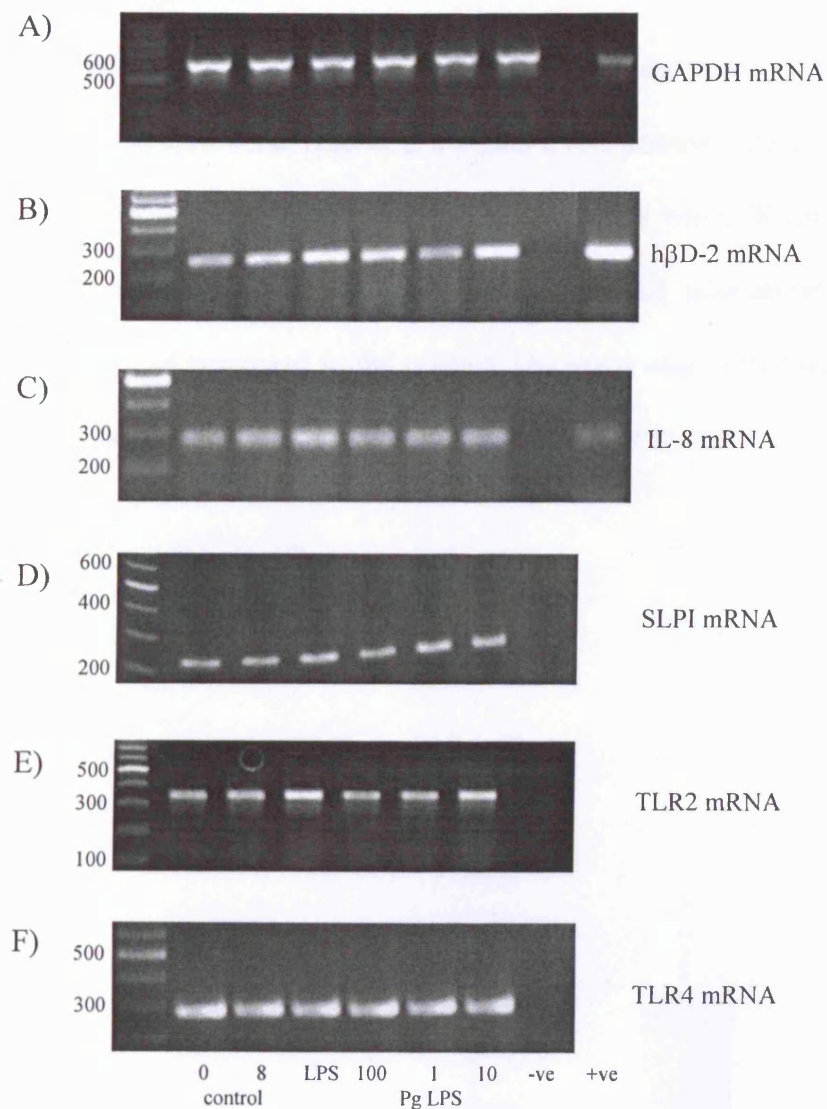


Figure 5.6 RT-PCR to show relative levels of mRNA obtained from oral epithelial cells treated with 100ng/ml, 1μg/ml and 10μg/ml *P. gingivalis* LPS for 8h. Results show mRNA for a) the housekeeping gene GAPDH, b) hβD-2, c) IL-8, d) SLPI, e) TLR2 and f) TLR4

5.4.4 The effect of *Streptococcus sanguis* lipoteichoic acid (LTA) on oral epithelial cells

5.4.4.1 IL-8 protein

A small increase in IL-8 protein secretion from oral epithelial cells was seen after stimulation with 100ng/ml and 1µg/ml *S. sanguis* LTA, however these increases were not found to be significant when analysed using the student's t-test. When stimulated at the higher concentration of 10µg/ml *S. sanguis* LTA, epithelial cells secreted a significantly higher amount of IL-8 compared to the control. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

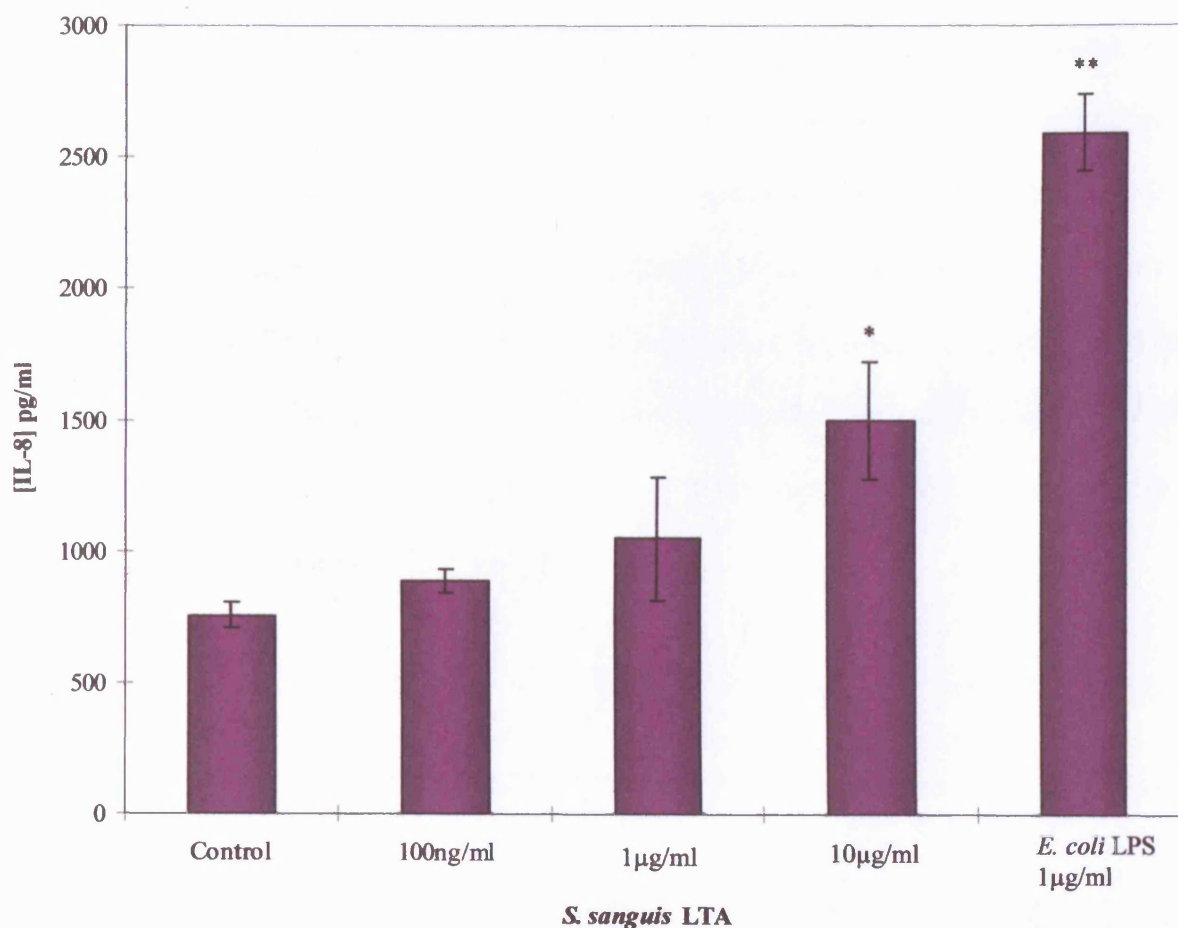


Figure 5.7 IL-8 production from oral epithelial cells following treatment with increasing concentrations of *S. sanguis* LTA and 1µg/ml *E. coli* LPS for 8h. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures * $p < 0.05$ ** $p < 0.005$

5.4.4.2 Messenger RNA

After stimulation with all concentrations of *S. sanguis* LTA, oral epithelial cells showed constitutive expression of IL-8 and TLR2 mRNA.

Gel photos for hβD-2, SLPI and TLR4 were further analysed using Phoretix software and the level of GAPDH in samples were compared to mRNA for each target. Results showed increased expression of hβD-2 mRNA after oral epithelial cells were stimulated with 100ng/ml and 10μg/ml *S. sanguis* LTA compared to the control. The concentration effect of *S. sanguis* LTA on oral epithelial cell hβD-2 mRNA highlights potential problems in the methods used. Optimisation of PCR, possibly with the reduction of PCR cycles may better demonstrate the effects of *S. sanguis* LTA on oral epithelial cells. Expression of mRNA in all samples was fairly low and any differences in processing of the samples may be exacerbated in the end results. When exposed to *S. sanguis* LTA, oral epithelial cells responded in a dose dependent fashion, with increasing production of SLPI mRNA at increasingly higher concentrations of *S. sanguis* LTA. Similar results were seen after analysis of TLR4 message, increasing concentrations of *S. sanguis* LTA, used to stimulate oral epithelial cells, led to the increasing upregulation of TLR4 mRNA, compared to the control. The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

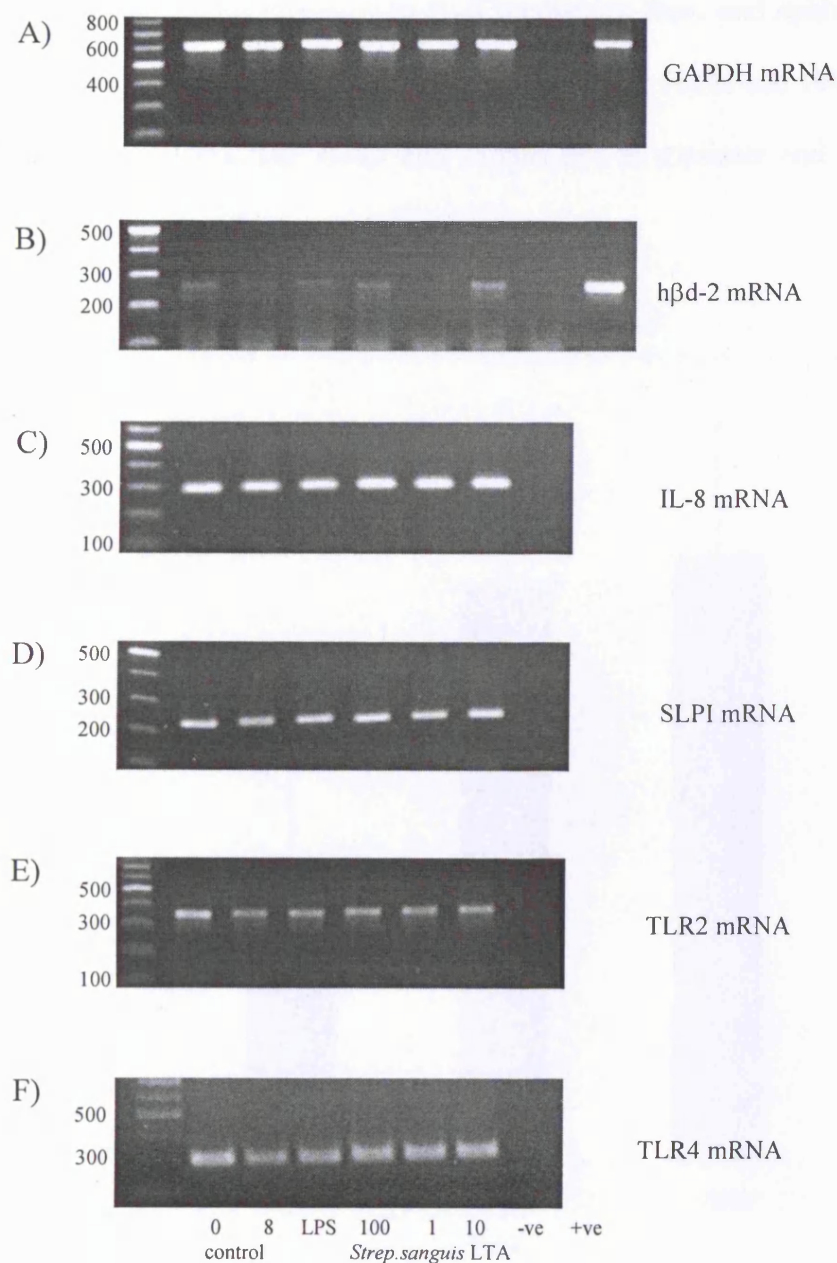


Figure 5.8 RT-PCR to show relative levels of mRNA obtained from oral epithelial cells treated with 100ng/ml, 1μg/ml and 10μg/ml *S. sanguis* LTA for 8h. Results show mRNA for a) the housekeeping gene GAPDH, b) hβD-2, c) IL-8, d) SLPI, e) TLR2 and f) TLR4

5.4.5 The effect of PMA on oral epithelial cells

5.4.5.1 IL-8 protein production

Cell monolayers were incubated with increasing doses, 1nM, 10nM and 100nM of the cell activator PMA for a period of 8 hours, for results see figure 4.10. All concentrations of PMA stimulated significant increases in IL-8 production from oral epithelial cells. After analysis using the student's t-test only the increases seen at 10nM and 100nM were shown to be significant ($p < 0.05$). The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

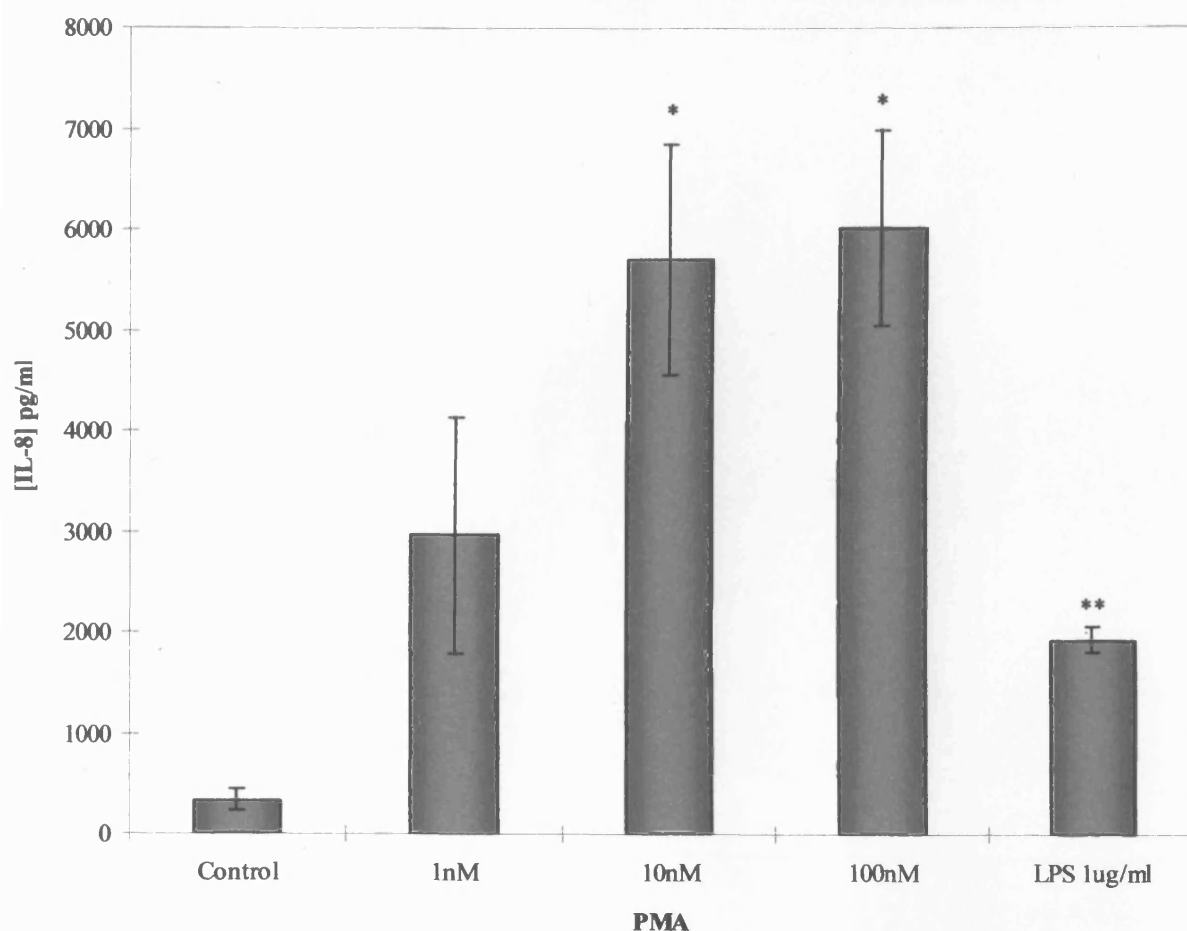


Figure 5.9 IL-8 production from oral epithelial cells following treatment with increasing concentrations of PMA and 1 µg/ml *E. coli* LPS for 8h. IL-8 levels in the culture supernatants were determined by ELISA and are expressed as mean \pm standard deviation of triplicate cultures * $p < 0.05$ ** $p < 0.005$

5.4.5.2 Messenger RNA

After stimulation with PMA at concentrations of 1, 10 and 100nM and 1µg/ml *E. coli* LPS, oral epithelial cells expressed the same levels of mRNA for GAPDH, SLPI, TLR2 and TLR4 compared to the control cells (Figure 4.11). After stimulation with PMA at all concentrations and 1µg/ml *E. coli* LPS, oral epithelial cells expressed greater levels of hβD-2 mRNA compared to control cells. This was also the case with IL-8 mRNA, the higher concentrations of 10 and 100nM PMA and *E. coli* LPS at a concentration of 1µg/ml stimulated increased expression of IL-8 in oral epithelial cells compared to control cells (Figure 4.11). The assay was carried out in triplicate and repeated; results shown are representative of repeated experiments.

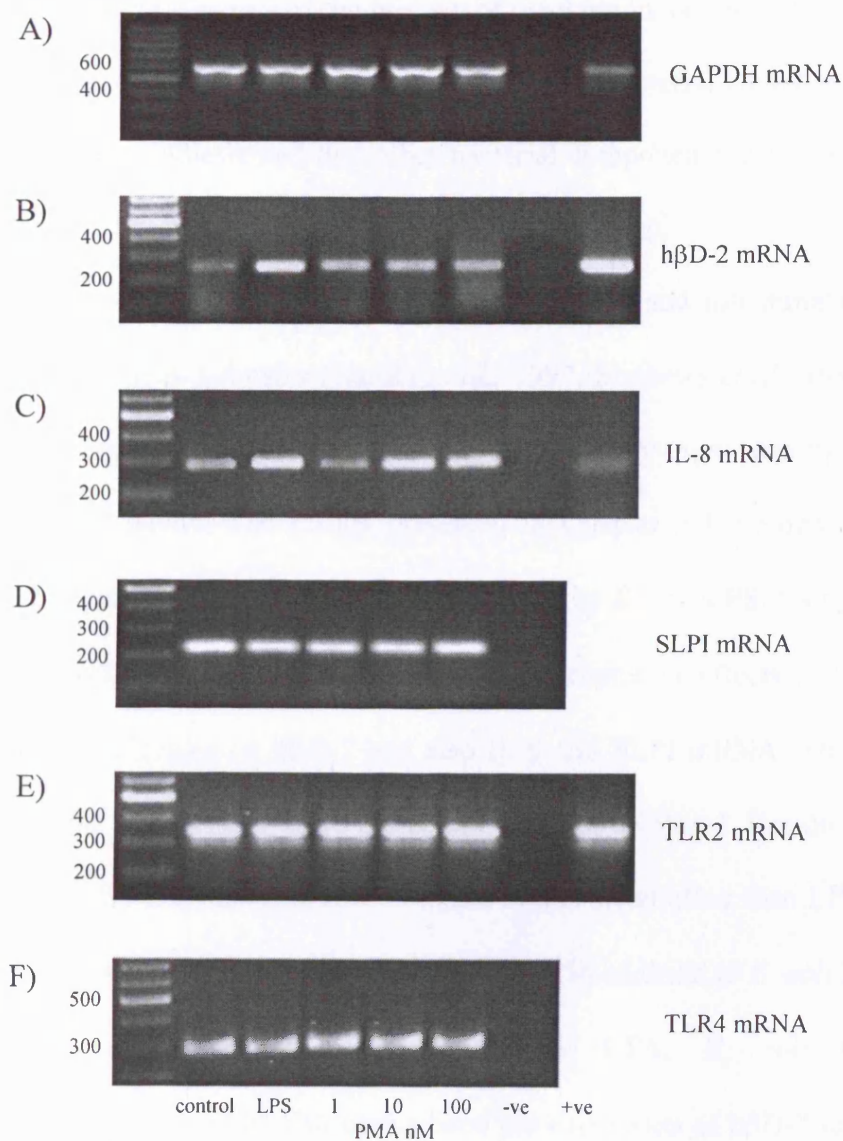


Figure 5.10 RT-PCR to show relative levels of mRNA obtained from oral epithelial cells treated with 1nM, 10nM and 100nM PMA for 8h. Results show mRNA for a) the housekeeping gene GAPDH, b)hβD-2, c)IL-8, d)SLPI, e)TLR2 and f)TLR4

5.5 Discussion

The use of endotoxins and lipopolysaccharides (LPS) over the last three decades has contributed to our understanding of the biology of cytokine induction in human cells. It is now well established that endotoxin/LPS is not the only bacterial component capable of stimulating cytokine synthesis and that other bacterial components and secreted products also have the capability to stimulate cytokine gene transcription.

It has been shown that exposure of epithelial cells to LPS and inflammatory mediators induce the synthesis of β -defensins (Harder *et al.*, 1997, Mathews *et al.*, 1999, Liu *et al.*, 2002). However, few studies have examined the induction by organisms that are directly relevant to the oral cavity. The results presented in Chapter 3 have demonstrated that h β D-2 mRNA is readily induced in oral epithelial cells by *E. coli* LPS. Using *E. coli* LPS as a positive control further investigation sought to determine the effects of other bacterial pro-inflammatory molecules on h β D-2 and also IL-8 and SLPI mRNA. The presence of mRNA for possible receptors of these molecules was also explored. Results presented in this chapter report for the first time that bacterial components other than LPS upregulate the production of h β D-2 mRNA in oral epithelial cells. In addition to *E. coli* LPS, the cell stimulant PMA, *P. gingivalis* LPS, *S. sanguis* LTA, *E. coli* GroEL and *A. actinomycetemcomitans* SAM also upregulated the expression of h β D-2 mRNA in oral epithelial cells. It was also determined that *A. actinomycetemcomitans* SAM and *E. coli* GroEL stimulated increased production of IL-8 from oral epithelial cells, however, this effect was negated after cells were pre-incubated with Polymyxin B. In the case of *E. coli* GroEL, the highest concentration of 10 μ g/ml also induced an increase in IL-8 mRNA; however, much of this was also abrogated by pre-incubation of epithelial cells with Polymyxin B. The cell stimulant PMA and *S. sanguis* LTA also induced the production of IL-8 from oral epithelial cells, both in a dose dependent fashion. Whilst *S. sanguis* LTA had no effect on IL-8 mRNA, PMA also induced IL-8 mRNA at all concentrations.

P. gingivalis LPS failed to elicit any response from cells with regard to IL-8 protein or mRNA. Densitometry suggests that *P. gingivalis* LPS and *S. sanguis* LTA, to a small degree, upregulated the mRNA for SLPI in oral epithelial cells, and *A. actinomycetemcomitans* SAM downregulated the expression of SLPI mRNA. However, since the results are not quantitative further research would be needed to confirm this upregulation in oral epithelial cells.

The results in Chapter 4 also demonstrated that the oral epithelial cell line H357 expresses mRNA for both TLR2 and TLR4. Despite the fact that TLR4 is an established receptor for LPS, stimulation with *E. coli* LPS proved to have little effect on the levels of mRNA for these receptors. Since results have subsequently shown that in addition to *E. coli* LPS other bacterial components possess the ability to up regulate both the chemokine IL-8 and h β D-2; it was interesting to establish the effect these factors would have on TLR2 and TLR4 mRNA. However, although results showed that *P. gingivalis* LPS appears to slightly upregulate TLR2 mRNA and *S. sanguis* LTA had a similar effect on TLR4 mRNA, the results are not quantitative and further research would be needed to confirm this upregulation in oral epithelial cells.

The results in this chapter lend more evidence to the growing theory that LPS may not be the major component of bacteria stimulating innate immune factors from host cells. Since these studies, others have also demonstrated the effects of both commensal and pathogenic bacteria on oral epithelial cells.

For the first time, results have shown that SAM from *A. actinomycetemcomitans* was able to stimulate the production of h β D-2 mRNA from oral epithelial cells. Furthermore, two strains of *A. actinomycetemcomitans* (whether live or heat-killed) were found to stimulate an increase of h β D-2 mRNA expression in oral epithelial cells (Chapter 6). Heat killing of the *A. actinomycetemcomitans* strains has no effect on the ability of either strain to

stimulate increased expression of hβD-2 mRNA. As *A. actinomycetemcomitans* SAM is heat-sensitive, this shows that there may be more than one stimulatory factor responsible for the increased expression of hβD-2 mRNA in oral epithelial cells. It has been shown that the component of *A. actinomycetemcomitans* SAM which stimulates the release of IL-6 from human fibroblasts is sensitive to both heat and trypsin treatment (Reddi *et al.*, 1996).

Results presented herein suggest that this bacterium plays a role in stimulating immune responses in oral epithelial cells. Therefore hβD-2 may have an important role in the oral defence against this organism.

Previously, SAM from *A. actinomycetemcomitans*, *E. corrodens*, *P. gingivalis*, *P. intermedia* and *Campylobacter rectus* have been found to be capable of stimulating the release of several pro-inflammatory cytokines (IL-1, TNFα, and IL-6) from various human cell populations including monocytes, gingival fibroblasts, peripheral blood mononuclear cells (PBMC) and neutrophils (Reddi *et al.*, 1995 and Reddi *et al.*, 1996b). Results presented in this chapter show that *A. actinomycetemcomitans* SAM had no effect on the secretion of IL-8 from oral epithelial cells since pre-incubation of oral epithelial cells with the LPS neutralising compound Polymyxin B negates any IL-8 release from oral epithelial cells. This suggests that *A. actinomycetemcomitans* SAM is contaminated with LPS which may be responsible for stimulating the release of IL-8 from oral epithelial cells.

Studies in this chapter also show for the first time that stimulation of oral epithelial cells with *E. coli* GroEL results in greater levels of hβD-2 mRNA expression. When oral epithelial cells had been pre-incubated with Polymyxin B they were still stimulated to increase expression of hβD-2 mRNA, however the levels were not as great. This suggests that contamination with LPS was contributing to the increased expression of hβD-2 mRNA in oral epithelial cells. This is also true of IL-8 mRNA, whilst pre-incubation of oral epithelial cells with Polymyxin B resulted in cells which no longer responded to *E. coli*

GroEL with the production of IL-8 protein, the opposite was true with IL-8 mRNA. The highest concentration of GroEL tested, 10µg/ml, still induced expression of IL-8 mRNA, although to a lesser degree than oral epithelial cells subjected to no pre-incubation with Polymyxin B, as seen with hβD-2 mRNA.

Previous studies have shown that *E. coli* GroEL was able to induce the release of TNF-α, IL-1α, IL-6 and sICAM-1 from skin keratinocytes at concentrations ranging from 0.1µg/ml - 5µg/ml. Control experiments carried out using Polymyxin B to neutralise traces of LPS that could be present in HSP preparations gave the same results as HSPs used alone (Marcatill *et al.*, 1997). It has also been shown that hsp60 or the GroEL-like protein from *P. gingivalis* and *A. actinomycetemcomitans* have been implicated in the pathogenesis of periodontal diseases in terms of the induction of a humoral immune response (Koga *et al.*, 1993, Maeda *et al.*, 1994, Tabeta *et al.*, 2000). These results, and those presented in this chapter, further support the potential role of HSPs in modulating cell interactions during immunological and inflammatory responses in human cells.

Studies by Krisanaprakornkit *et al.* (2000) conducted in the same year as those presented in this chapter show that in the case of the oral commensal *F. nucleatum*, a cell wall extract induced gingival epithelial cells to synthesise hβD-2 mRNA, whilst extracts of *P. gingivalis* did not. Further, whilst a cell wall extract upregulated oral epithelial cell hβD-2 mRNA, *F. nucleatum* LPS did not. In contrast, results in this thesis have shown that both *P. gingivalis* LPS and whole bacteria, whether live or heat killed (results presented in Chapter 6), upregulated hβD-2 mRNA expression in oral epithelial cells.

Heating of *P. gingivalis* would not affect LPS, therefore *P. gingivalis* LPS would seem to be the major hβD-2 mRNA stimulating component of this bacterium. Further, the upregulation of hβD-2 mRNA is greater with *P. gingivalis* LPS than that seen with whole *P. gingivalis*, probably due to the LPS being more concentrated than when oral epithelial

cells are incubated with whole *P. gingivalis* cells. Results in this chapter also showed for the first time the inability of *P. gingivalis* LPS to upregulate IL-8 protein in oral epithelial cells.

Previously it has been shown that whole *P. gingivalis* was able to strongly inhibit IL-8 accumulation from gingival epithelial cells. Inhibition was associated with a decrease in mRNA for IL-8 (Darveau *et al.*, 1998). Also, *P. gingivalis* LPS has been shown to be a transcriptional inhibitor of IL-8 and E-selectin expression in human vascular umbilical cord endothelial cells (HUVEC) (Darveau *et al.*, 1995). It has been shown that the expression of a trypsin-like protease activity was related to decreased extracellular levels of IL-6 and IL-8 from human gingival fibroblasts (Steffen *et al.*, 2000).

In addition to Gram-negative bacterial products from oral pathogens, it has been shown, for the first time, that *S. sanguis* LTA also increased the expression of IL-8 protein and hβD-2 mRNA in oral epithelial cells. Previous studies have reported that *S. aureus* LTA elicited a time and concentration dependent release of TNF-α, IL-1β, IL-6 and IL-8 in whole blood and primary adherent monocytes. Messenger RNA encoding TNF-α, IL-1β and IL-6 seemed to be accumulated in monocytes and T-cells, but not in granulocytes and B-cells (Ellingsen *et al.*, 2002).

SLPI functions in the protection of mucosal surfaces from degradation by proteases during inflammation (Fink *et al.*, 1986, Thompson *et al.*, 1986, Ohlsson *et al.*, 1988) also; human SLPI displays broad-spectrum antibacterial activity (Hiemstra *et al.*, 1996). Studies have also found SLPI to be an LPS-induced IFN gamma-suppressible phagocyte product that serves to inhibit LPS responses (Jin *et al.*, 1997).

LTA derived from *Staph. aureus* induces SLPI in macrophages (Jin *et al.*, 1998). During inflammation induced by bacteria or their products, SLPI may be induced both directly and

by anti-inflammatory cytokines, some of whose actions it may mediate. These newly described inducers of SLPI (IL-10, IL-6 and LTA) and the kinetics of its induction (direct and indirect, slow to rise then prolonged) are both consistent with the hypothesis that SLPI may act in an autocrine fashion as a brake on the response of macrophages to microbial inflammation.

In human epithelial cells, SLPI expression was induced by TNF- α and LPS at concentrations that could be considered supraphysiologic (10 μ g/ml) (Maruyama *et al.*, 1994, Sallenave *et al.*, 1994).

Human α -defensins have been shown to significantly increase SLPI protein release by PBMCs in a time and dose-dependent fashion without affecting SLPI mRNA synthesis (Van Wetering *et al.*, 2000). Since both *S. sanguis* LTA and *P. gingivalis* LPS may upregulate SLPI, although results in this thesis have failed to show this convincingly, it could be suggested that any possible induction is as a result of stimulation directly by these bacterial products or by increased levels of IL-8 or h β D-2, which, as demonstrated in this chapter, are also induced by these bacterial products.

Data presented in this chapter have shown that h β D-2 mRNA is induced in oral epithelial cells by both commensal and pathogenic bacteria. Studies presented in this chapter therefore fail to demonstrate a clear-cut relationship between pathogenic and commensal oral bacteria and their interactions with human β -defensins.

The results presented in this chapter are in agreement with previous results where the mammalian mucosal antimicrobial peptide TAP mRNA levels were increased in TEC upon challenge with heat-killed bacteria, bacterial outer membrane components, and certain inflammatory cytokines (Diamond *et al.*, 1996, Russell *et al.*, 1996, Diamond *et al.*, 2000). Other mucosal epithelial sites have demonstrated a similar pattern, with h β D-2 mRNA being induced in gastric epithelial cells and skin epithelial cells by both pathogenic and

commensal bacteria (O'Neil *et al.*, 1999, 2000, Dinulos *et al.*, 2003). Evidence suggests that h β D-2 plays an important role in mucosal innate immunity and it appears to be a constant presence and has broad-spectrum antibacterial activity. When exposed to pathogenic bacteria, the role of h β D-2 may change and become an important part of the adaptive immune response. Recent studies have shown that using chemokine receptors on dendritic cells and T cells, defensins might also contribute to the regulation of host adaptive immunity against microbial invasion (Yang *et al.*, 1999, 2002). Defensins have considerable immunological adjuvant activity and linkage of beta-defensins or selected chemokines to an idiotypic lymphoma antigen has yielded potent antitumor vaccines (Biragyn *et al.*, 2001).

Another important property of antimicrobial peptides is their ability to bind avidly to potentially pro-inflammatory molecules released from micro-organisms, such as LPS, LTA and DNA (Devine, 2003). By binding these molecules, antimicrobial peptides inhibit responses of host cells and damp-down an undesirable inflammatory response (Scott *et al.*, 1999, 2000a, b, Nagaoka *et al.*, 2001). Results in this chapter have shown that several microbial products induce h β D-2 mRNA. Increased levels of h β D-2 may, therefore, contribute to preventing an undesirable inflammatory response by binding the very molecules that have induced their production.

Oral epithelial cell activation by all of these bacterial components is presumably via specific binding to cell surface receptors and activation of intracellular transduction pathways leading to the generation of h β D-2 gene transcription factors in the nucleus.

Multiple types of TLR have been described on mammalian cells and so it has been proposed that the innate immune system may use different combinations of TLRs to recognise different groups of microbial pathogens. For example, TLR2 is activated by Gram-positive bacterial derived products including lipoproteins and peptidoglycan in

macrophages (Schwandner *et al.*, 1999). Results have shown that TLR4-deficient mice demonstrate hyporesponsiveness to LPS, showing that TLR4 is a critical receptor for LPS signalling (Lemaitre *et al.*, 1996).

There have recently been published studies which link mammalian TLR activation and the induction of antimicrobial peptides. In recent years the studies have identified the expression of both CD14 and TLRs in human tracheal epithelial cells (Becker *et al.*, 2000). At least two of the identified TLRs have been implicated as functioning in conjunction with CD14 to transduce the signal from microorganisms (Kirschning *et al.*, 1998, Chow *et al.*, 1999).

Results in Chapter 4 showed that whilst the oral epithelial cell line H357 expressed no detectable CD14 on their surface, CD14 was necessary for the upregulation of IL-8 protein and h β D-2 mRNA. Results have also shown the presence of TLR2 and TLR4 mRNA in H357 cells, and whilst studies in this chapter show that the bacterial factors tested had no effect on the expression of TLR2 and TLR4, there may still be a possible role for TLRs in the upregulation of h β D-2 mRNA in oral epithelial cells.

Unlike *E. coli* LPS, *P. gingivalis* LPS has been shown to act through TLR2 rather than TLR4, this may be a reason for the upregulation of TLR2 mRNA by *P. gingivalis* LPS in oral epithelial cells. The lack of TLR-4 response to *P. gingivalis* LPS is consistent with the observation that LPS non-responder mice, which have a defect in TLR4 typically do not respond differently than wild-type mice to *P. gingivalis* LPS (Hanazawa *et al.*, 1985, Takada *et al.*, 1990, Tanamoto *et al.*, 1997) and shows that *P. gingivalis* LPS uses different TLRs for activation of host cells (Ernst *et al.*, 1999, Netea *et al.*, 2002, Ogawa *et al.*, 2002). The five branched acyl chain and mono-phosphorylated lipid A of *P. gingivalis* differs substantially from the typical hexa-acyl diphosphorylated *E. coli* lipid A like template recognised by TLR4. Further, it is interesting to note that LPS extracted from

B. fragilis has already been shown capable of activating TLR4 mutant mouse (C3H/HeJ) cells and shares a lipid A with striking resemblance to that of *P. gingivalis* lipid A (Delahooke *et al.*, 1995).

Further, it has been shown that IL-8 production in HGEC stimulated with *P. gingivalis* fimbrial protein was clearly inhibited by mouse monoclonal antibody to human TLR2. These findings suggest that *P. gingivalis* fimbrial protein and its active peptide are capable of activating HGEC through TLR2 (Asai *et al.*, 2001). Another study has shown that *P. gingivalis* is unusual in that it engages TLR2 and not TLR4 when examined in stably transfected CHO cell lines (Bainbridge and Darveau, 2001).

After the identification of TLRs, a number of controversial reports of the involvement of TLR2, TLR4 and MD-2 in LTA-induced cell activation have been published (Schwander *et al.*, 1999, Takeuchi *et al.*, 2000, Dziarski *et al.*, 2001, Nagai *et al.*, 2002). However, although LTA is meanwhile regarded as an important mediator of inflammation (Ginsburg *et al.*, 2002), the recruitment of TLRs is still unclear, because in most of these studies commercial preparations of LTA were used (Bhakdi *et al.*, 1991, Keller *et al.*, 1992, Schwander *et al.*, 1999 Nagai *et al.*, 2002) which not only display a high degree of compositional heterogeneity but are contaminated by significant amounts of LPS (Gao *et al.*, 2001, Morath *et al.*, 2001). Whilst others have suggested the involvement of TLR2 (Schwander *et al.*, 1999, Yoshimura *et al.*, 1999, Lehner *et al.*, 2001 Opitz *et al.*, 2001) *S. sanguis* LTA induced IL-8 secretion in primed HSC-2 cells was inhibited by anti TLR4 MAb to the control level, but not by anti TLR2 MAb (Uehara *et al.*, 2002). The ability of LTA to act through TLR4 may explain the capability of this molecule to upregulate TLR4 mRNA in oral epithelial cells.

Results published since these studies were conducted have further demonstrated the importance of TLRs in the stimulation of cells to express h β D-2. HEK293 cells transfected with TLR2, but not wild type cells responded to stimulation with bacterial lipoprotein by production of h β D-2. Furthermore, the human lung epithelial cell line A549 was found to constitutively express TLR2 and express h β D-2 in response to lipoprotein stimulation. This response was abrogated by blocking the signalling pathway activated through TLRs by transfecting the cells with a dominant-negative form of IRAK-2 (Birchler *et al.*, 2001).

TLR-dependent production of h β D-2 is likely not unique to airway epithelial cells, but rather a host defense mechanism common to many epithelial cell sites in the body. Expression of TLRs and h β D-2 has been demonstrated in various epithelial cell types. For example, primary gingival epithelial cells are activated when exposed to bacterial fimbriae (Asai *et al.*, 2001) and exposure to LPS, which is known to activate monocytes via TLR4 (Poltorak *et al.*, 1998), results in increased h β D-2 in gingival keratinocytes (Mathews *et al.*, 1999) and oral epithelial cell lines, as shown in this chapter. In addition, primary keratinocytes expressing TLR2 mRNA and protein have been shown to respond to peptidoglycan, a TLR2 agonist (Yoshimura *et al.*, 1999), by upregulation of h β D-2 mRNA and IL-8 protein (Kawai *et al.*, 2002), although TLR2-dependence was not demonstrated. Overall, it seems likely that TLR engagement and h β D-2 production will be linked in these and other cell types.

Chapter 6

Epithelial cell IL-8 and hβD-2 synthesis in response to intact bacteria

Chapter 6

Epithelial cell IL-8 and h β D-2 synthesis in response to intact bacteria

6.1 Introduction

In life, the epithelial cells of the oral mucosa will be exposed to a range of stimulatory bacterial components. Amongst such components will be the intact bacteria themselves. Such bacteria may be both viable and metabolically active, or non-viable. In this chapter, oral epithelial cells were co-cultured with various multiplicities of infection of the range of Gram-negative and Gram-positive organisms that they are likely to encounter on a daily basis. The bacteria were presented as living organisms or as dead cells in order to determine what effect they have on two key host cell outputs i.e. the potent neutrophil chemokine IL-8 and the antibacterial peptide, h β D-2.

6.2 Aims

To establish the role of viable or non-viable oral commensals and periodontopathogens in host innate immune protection by oral keratinocytes.

6.3 Materials and methods

The oral epithelial cell line H357 was seeded into 6-well plates (see Chapter 2, section 2.10). Subsequently, confluent oral epithelial cell monolayers were incubated with live or heat-killed bacteria grown to mid-exponential phase. Bacterial strains used in this chapter were *S. gordonii* DL1 Challis, *S. sanguis* NCTC 10904, *P. gingivalis* W50, *A. actinomycetemcomitans* SUNY 462 and *A. actinomycetemcomitans* SUNY 465. Bacteria were grown and prepared as described in Chapter 2, section 2.10. Oral epithelial cell monolayers were also exposed to 1µg/ml *E. coli* LPS. Studies in chapter 4, sections 4.4.1 and 4.4.2 demonstrated the upregulation of both IL-8 protein and hβD-2 mRNA by *E. coli* LPS in these cells.

After a 5h time period, 1ml of supernatant was removed and centrifuged at 8000rpm for 5 minutes to remove any bacteria present. Supernatant samples were then stored at -70°C for assay of IL-8 concentration by ELISA as described in section 2.3. Cell monolayers were washed twice with PBS and total cellular RNA extracted as described in section 2.4 for cells. Samples were then reverse transcribed, as described in section 2.5. 26 cycles of PCR were performed for GAPDH and 35 cycles for hβD-2 and IL-8, primers and programs were as described in Table 2. 20µl of PCR product was loaded and run on a 2% agarose gel as described in section 2.7.

6.4 Results

6.4.1 The effect of live and heat-killed oral bacteria on oral epithelial cells

6.4.1.1 IL-8 protein production

Oral epithelial cell monolayers were incubated with live and heat-killed *S. gordonii*, *S. sanguis*, *P. gingivalis* and two *A. actinomycetemcomitans* strains for 5 hours, and cell supernatant analysed for IL-8 protein concentration. Oral epithelial cell monolayers were also exposed to 1 µg/ml *E. coli* LPS to demonstrate a positive upregulation.

In each experiment, incubation of oral epithelial cells (OECs) with 1 µg/ml *E. coli* LPS led to an increase in the production of IL-8.

After incubation with live and heat-killed *S. gordonii* at an MOI of 10:1, there was an increase in the amount of IL-8 secreted from OECs (Figure 6.1). This increase was more marked after incubation with heat-killed *S. gordonii*, where the result was found to be statistically significant ($p < 0.05$) after analysis using the student's t-test (Figure 6.1). There was a small decrease in the amount of IL-8 protein secreted from OECs after incubation with live *S. gordonii* DL1 Challis at an MOI of 100:1 compared to the control, however, this result was found not to be significant using the Student's t-test (Figure 6.1).

Live *S. sanguis* stimulated a decrease in production of IL-8 protein from OECs at an MOI of 100:1 compared to the control, this was found to be statistically significant ($p < 0.005$) using the student's t-test (Figure 6.2). At the lowest MOI of 1:1, using live *S. sanguis*, there was a doubling in IL-8 production from OECs compared to the control, however, this was found not to be significant when analysed using the student's t-test. Live *S. sanguis*, when incubated with OECs at an MOI of 10:1 had no effect on oral epithelial cell IL-8 production. At all MOIs, heat-killed *S. sanguis* had no significant effect on the production of IL-8 from OECs (Figure 6.2).

After incubation with live *P. gingivalis* at an MOI of 1:1, OECs produced significantly ($p < 0.005$) more IL-8, compared to the control (Figure 6.3). However, increasing the MOI

to 10 and 100:1 had no effect on OECs, with stimulated OECs producing the same level of IL-8 compared to the control. Heat-killed *P. gingivalis* at all MOIs induced OECs to produce a greater amount of IL-8 compared to the control. At an MOI of 1 and 100:1 these results were found to be statistically significant ($p < 0.05$) when analysed using the student's t-test (Figure 6.3).

Live and heat-killed *A. actinomycetemcomitans* SUNY 462, at MOIs of 10 and 100:1 stimulated OECs to produce significantly ($p < 0.005$ and $p < 0.0005$) greater levels of IL-8 compared to the control (Figure 6.4).

After incubation with live and heat-killed *A. actinomycetemcomitans* SUNY 465 for 5 hours at all MOIs, OECs were induced to produce significantly ($p < 0.05$ and $p < 0.005$) greater levels of IL-8 compared to the control (Figure 6.5). The increase in IL-8 production was greater after incubation with live *A. actinomycetemcomitans* SUNY 465 compared to heat-killed *A. actinomycetemcomitans* SUNY 465 (Figure 6.5).

All assays were carried out in triplicate and repeated; results shown are representative of repeated experiments.

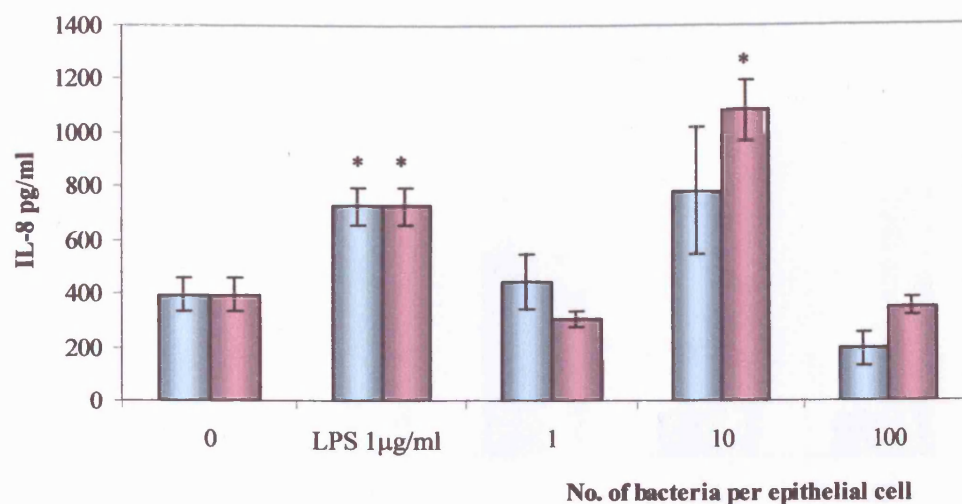


Figure 6.1 Production of IL-8 from the oral epithelial cells after incubation with live and heat-killed *S. gordonii* for 5 hours. ■ live *S. gordonii* ■ heat-killed *S. gordonii*. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk. * $P < 0.05$

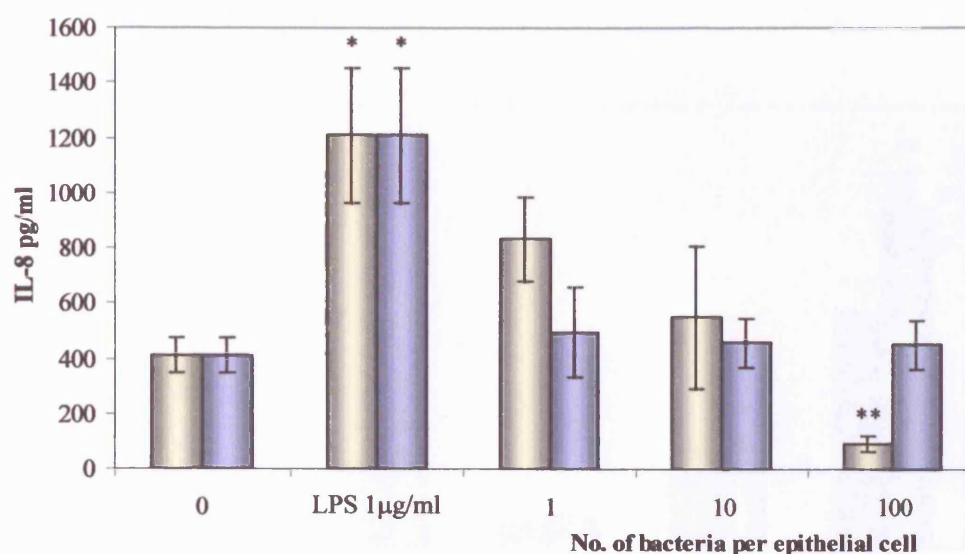


Figure 6.2 Production of IL-8 from the oral epithelial cells after incubation with live and heat-killed *S. sanguis* for 5 hours. ■ live *S. sanguis* ■ heat-killed *S. sanguis*. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk. * $p < 0.05$ ** $p < 0.005$

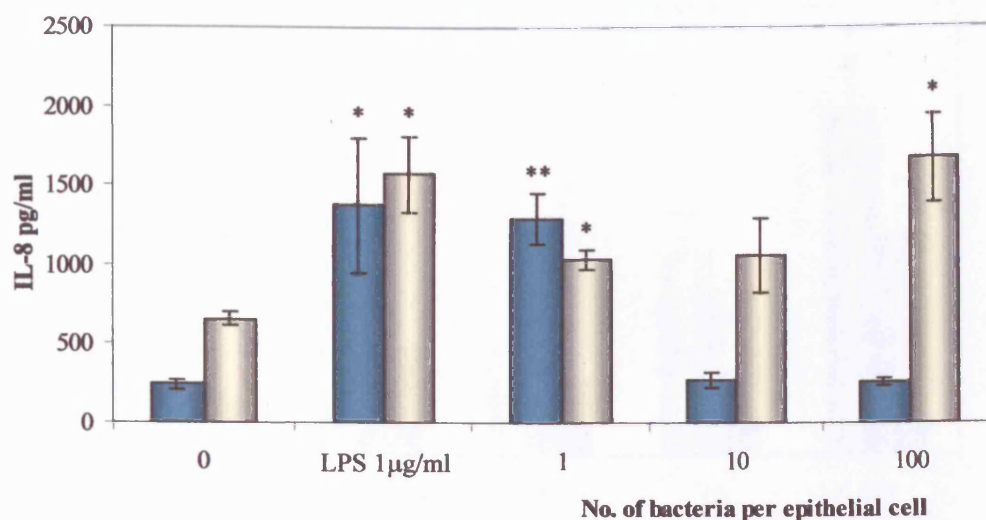


Figure 6.3 Production of IL-8 from the oral epithelial cells after incubation with live and heat-killed *P. gingivalis* for 5 hours ■ live *P. gingivalis* □ heat-killed *P. gingivalis*. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk. * $p < 0.05$ ** $p < 0.005$

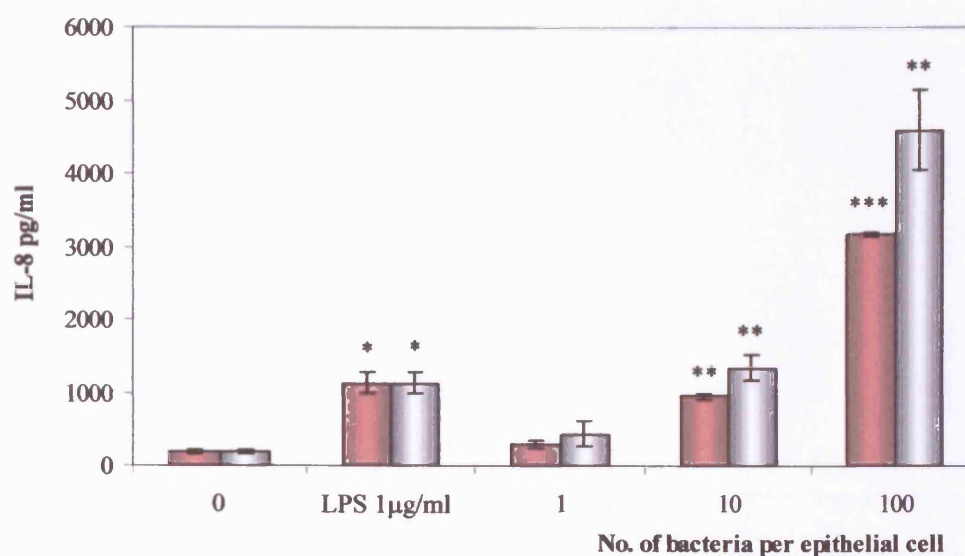


Figure 6.4 Production of IL-8 from the oral epithelial cells after incubation with live and heat-killed *A. actinomycetemcomitans* SUNY 462 for 5 hours ■ live *A. actinomycetemcomitans* SUNY 462 □ heat-killed *A. actinomycetemcomitans* SUNY 462. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk. * $p < 0.05$ ** $p < 0.005$ *** $p < 0.0005$

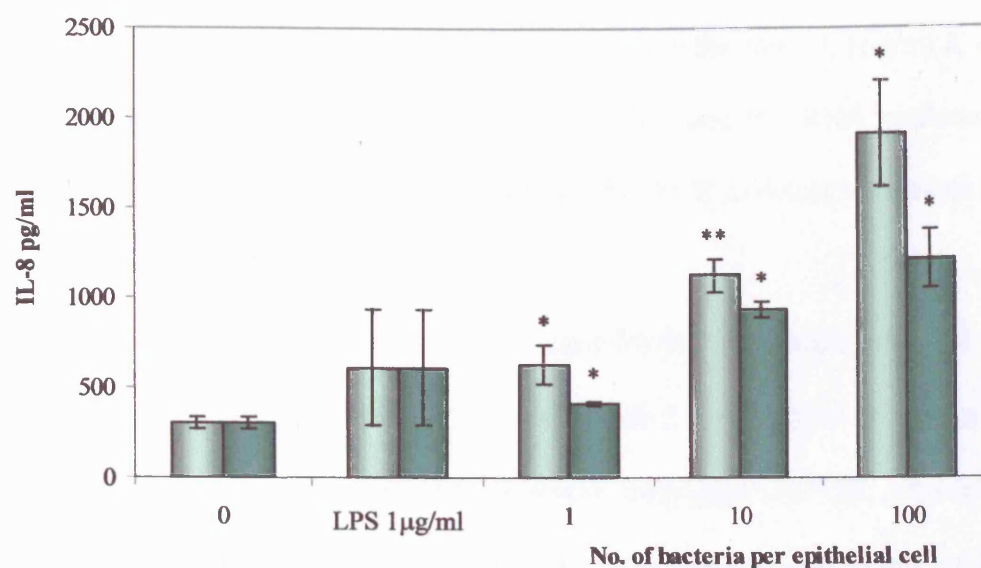


Figure 6.5 Production of IL-8 from the oral epithelial cells after incubation with live and heat-killed *A. actinomycetemcomitans* SUNY 465 for 5 hours

■ live *A. actinomycetemcomitans* SUNY 465

■ heat-killed *A. actinomycetemcomitans* SUNY 465

Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk. * $p < 0.05$ ** $p < 0.005$

6.4.1.2 Messenger RNA

After exposure to live or heat-killed oral bacteria and the control, 1µg/ml *E. coli* LPS, for 5 hours, oral epithelial cell monolayers were lysed and the RNA extracted and reverse transcribed. The resulting cDNA was subjected to PCR to detect transcripts for GAPDH, a housekeeping gene, hβD-2 and IL-8.

After 5 hours, control oral epithelial cell monolayers, those exposed to all concentrations of live and heat-killed bacteria at all MOIs and those exposed to 1µg/ml *E. coli* LPS, showed constitutive expression of the housekeeping gene GAPDH. All oral epithelial cell monolayers exposed to 1µg/ml *E. coli* LPS expressed greater levels of hβD-2 mRNA compared to control cells (Figures 6.6 to 6.10).

The levels of mRNA for hβD-2 detected in oral epithelial cell monolayers were slightly increased after exposure to live *S. gordonii* at an MOI of 1:1 and 10:1 compared to control cells (Figure 6.6). At the highest MOI of 100:1, live *S. gordonii* had no effect on the level of hβD-2 mRNA produced by OECs compared to the control. OECs incubated with heat-killed *S. gordonii* at all MOIs expressed the same level of hβD-2 mRNA as the control cells. After 5h incubation with 1µg/ml *E. coli* LPS, live and heat-killed *S. gordonii* at all MOIs, the levels of IL-8 mRNA were comparable to those seen in unstimulated, control cell monolayers (Figure 6.6).

When exposed to heat-killed *S. sanguis* at all MOIs, OECs were stimulated to express greater levels of hβD-2 mRNA compared to the control (Figure 6.7). OECs exposed to live *S. sanguis* at an MOI of 1:1 and 10:1 also expressed greater levels of hβD-2 mRNA compared to the control. After incubation with live *S. sanguis* at the highest MOI OECs expressed no hβD-2 mRNA, which is the same as control cells. The level of IL-8 mRNA after OECs had been incubated with 1µg/ml *E. coli* LPS and live or heat-killed *S. sanguis* at all MOIs was greater compared to the control.

OECs exposed to live and heat-killed *P. gingivalis* for 5 hours showed constitutive expression of IL-8 mRNA in all samples, including controls, which included unstimulated cells and OECs exposed to 1 µg/ml *E. coli* LPS (Figure 6.8). OECs expressed greater levels of hβD-2 mRNA when stimulated with live and heat-killed *P. gingivalis* at all MOIs, compared to controls. However, after stimulation with heat-killed *P. gingivalis* hβD-2 mRNA was induced to a lesser extent at the highest MOI of 100:1 (Figure 6.6).

After incubation with live *A. actinomycetemcomitans* SUNY 462 for 5 hours at an MOI of 10 and 100:1, OECs expressed greater levels of hβD-2 mRNA compared to the controls (Figure 6.9). The incubation of OECs with live *A. actinomycetemcomitans* SUNY 462 for 5 hours at all MOIs led to a slight decrease in IL-8 mRNA compared to the control. The level of hβD-2 mRNA expressed by OECs was increased by exposure to heat-killed *A. actinomycetemcomitans* SUNY 462 at all MOIs (Figure 6.9). However, greater induction of hβD-2 mRNA was seen after incubation with higher numbers of *A. actinomycetemcomitans* SUNY 462. As with live *A. actinomycetemcomitans* SUNY 462, the incubation of OECs with heat-killed *A. actinomycetemcomitans* SUNY 462 for 5 hours at all MOIs led to a slight decrease in IL-8 mRNA compared to the control (Figure 6.9).

Incubation of OECs with live *A. actinomycetemcomitans* SUNY 465 for 5 hours at all MOIs led to increased expression of hβD-2 mRNA compared to the control. Live *A. actinomycetemcomitans* SUNY 465, incubated with OECs at all MOIs for 5 hours, induced a slight decrease in IL-8 mRNA levels compared to the control (Figure 6.10). After 5 hours incubation with heat-killed *A. actinomycetemcomitans* SUNY 465, OECs produced greater levels of hβD-2 mRNA at all MOIs, although the increase was more marked when OECs were exposed to greater numbers of *A. actinomycetemcomitans* SUNY 465. As with live *A. actinomycetemcomitans* SUNY 465 at 5 hours, the incubation of

OECs with heat-killed *A. actinomycetemcomitans* SUNY 465 for 5 hours at all MOIs led to a slight decrease in IL-8 mRNA compared to the control (Figure 6.10).

All assays were repeated; results shown are representative of repeat experiments.

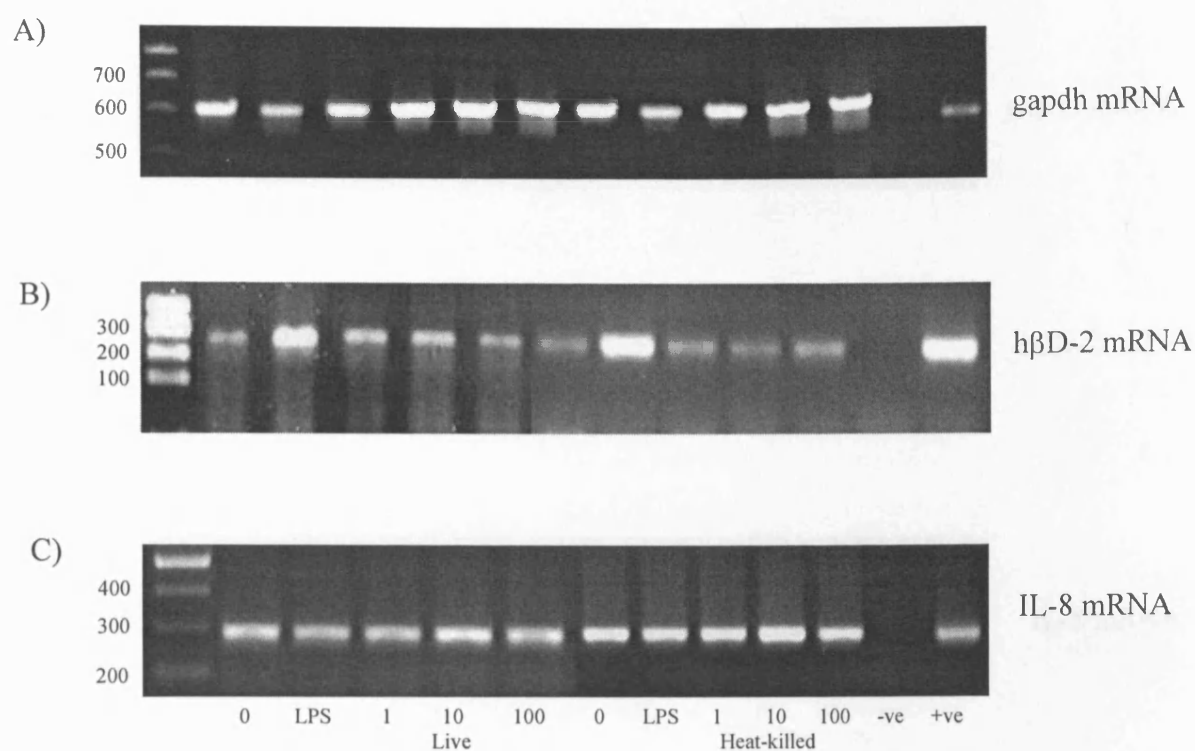


Figure 6.6 Relative levels of mRNA obtained from oral epithelial cells exposed to live and heat-killed *S. gordonii* for 5h. Results show mRNA for a) the housekeeping gene GAPDH, b) h β D-2 and c) IL-8. A DNA molecular marker was run in the first lane of the gel and is labeled above with the number of base pairs.

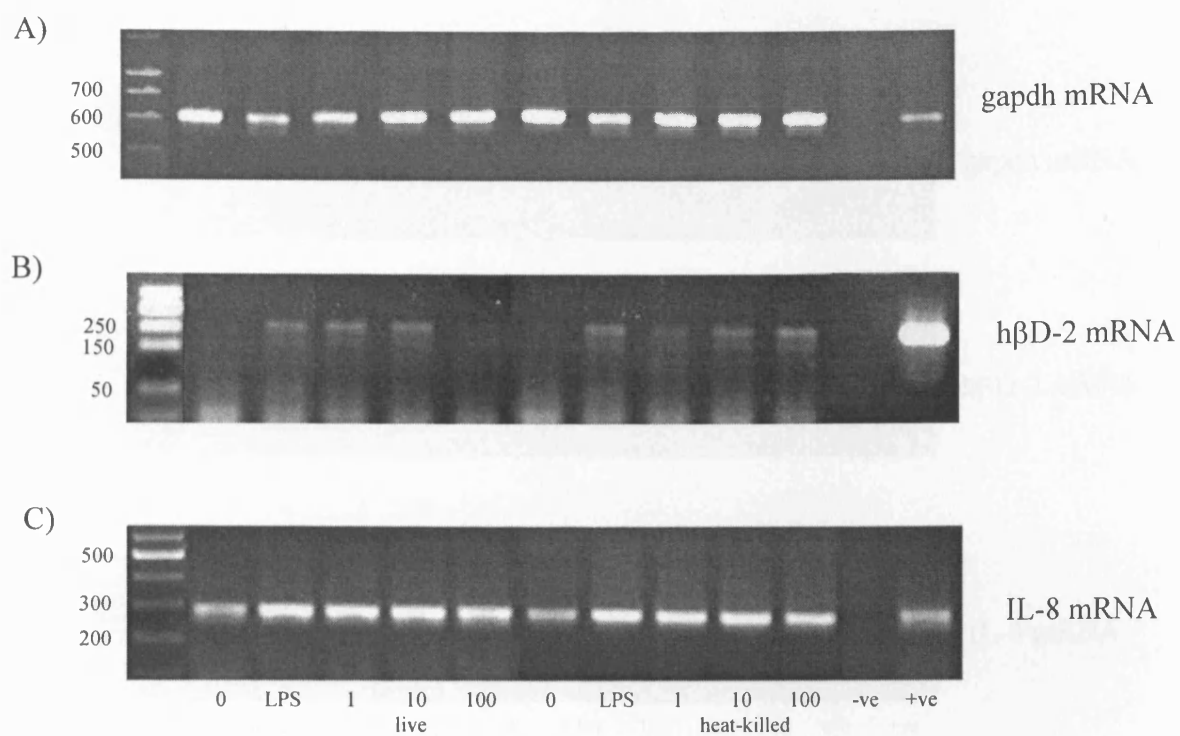


Figure 6.7 Relative levels of mRNA obtained from oral epithelial cells exposed to live and heat-killed *Strep. sanguis* for 5h. Results show mRNA for a) the housekeeping gene GAPDH, b) h β D-2, c) IL-8. A DNA molecular marker was run in the first lane of the gel and is labelled above with the number of base pairs

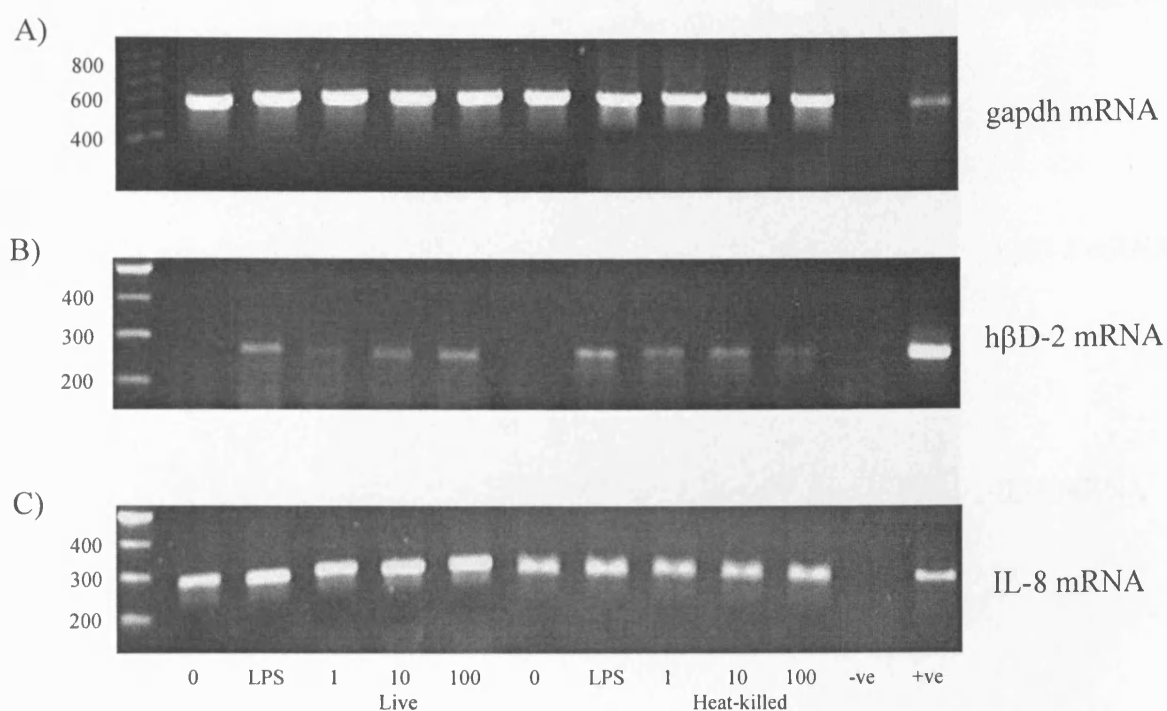


Figure 6.8 Relative levels of mRNA obtained from oral epithelial cells exposed to live and heat-killed *P. gingivalis* for 5h. Results show mRNA for a) the housekeeping gene GAPDH, b) h β D-2 and c) IL-8. A DNA molecular marker was run in the first lane of the gel and is labelled above with the number of base pairs.

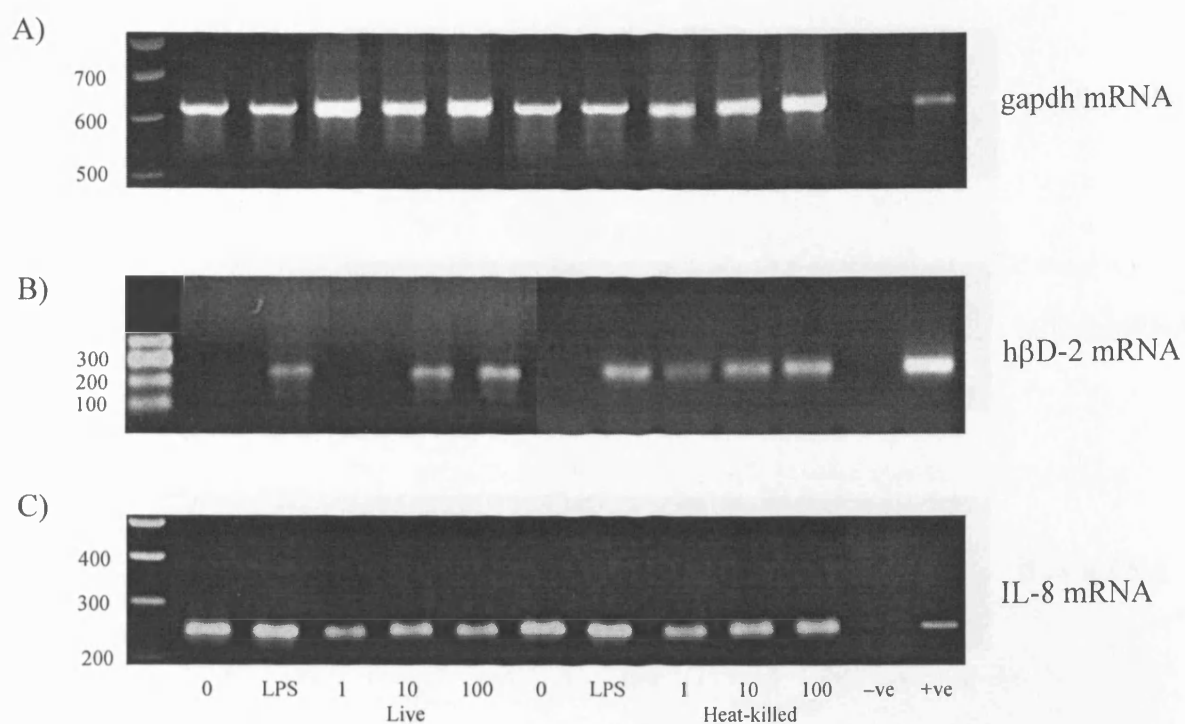


Figure 6.9 Relative levels of mRNA obtained from oral epithelial cells exposed to live and heat-killed *A. actinomycetemcomitans* SUNY 462 for 5h. Results show mRNA for a) the housekeeping gene GAPDH, b) h β D-2 and c) IL-8. A DNA molecular marker was run in the first lane of the gel and is labelled above with the number of base pairs.

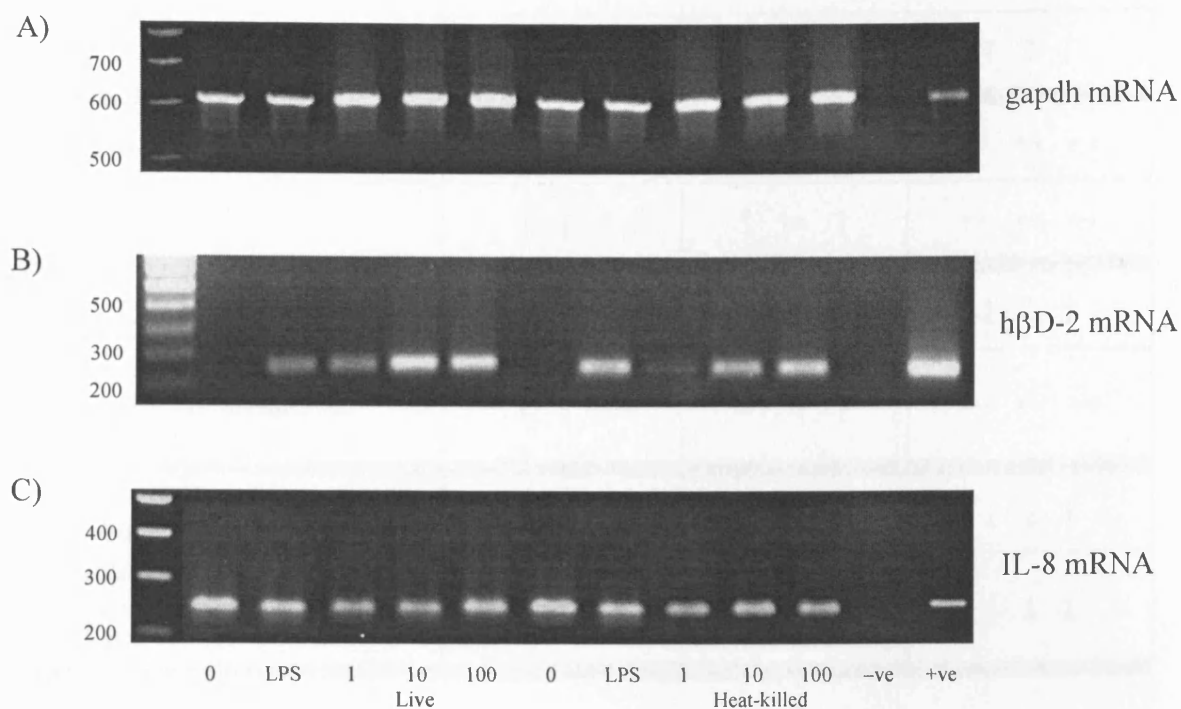


Figure 6.10 Relative levels of mRNA obtained from oral epithelial cells exposed to live and heat-killed *A. actinomycetemcomitans* SUNY 465 for 5h. Results show mRNA for a) the housekeeping gene GAPDH, b) h β D-2 and c) IL-8. A DNA molecular marker was run in the first lane of the gel and is labelled above with the number of base pairs.

6.4.2 Summary of results

Bacterial stimulation	hβD-2 mRNA	IL-8 protein	IL-8 mRNA
Live <i>S. gordonii</i>	↑ ↑ ↔	↔ ↑ ↔	↔ ↔ ↔
Heat-killed <i>S. gordonii</i>	↔ ↔ ↔	↔ ↑ ↔	↔ ↔ ↔
Live <i>S. sanguis</i>	↑ ↑ ↔	↑ ↔ ↓	↑ ↑ ↑
Heat-killed <i>S. sanguis</i>	↑ ↑ ↑	↔ ↔ ↔	↑ ↑ ↑
Live <i>P. gingivalis</i>	↑ ↑ ↑	↑ ↔ ↔	↔ ↔ ↔
Heat-killed <i>P. gingivalis</i>	↑ ↑ ↑	↑ ↔ ↑	↔ ↔ ↔
Live <i>A. actinomycetemcomitans</i> SUNY 462	↔ ↑ ↑	↔ ↑ ↑	↓ ↓ ↓
Heat-killed <i>A. actinomycetemcomitans</i> SUNY 462	↑ ↑ ↑	↔ ↑ ↑	↔ ↔ ↔
Live <i>A. actinomycetemcomitans</i> SUNY 465	↑ ↑ ↑	↑ ↑ ↑	↓ ↓ ↓
Heat-killed <i>A. actinomycetemcomitans</i> SUNY 465	↑ ↑ ↑	↑ ↑ ↑	↓ ↓ ↓

Table 6.1 Summary of results showing the effect of live and heat-killed oral bacteria on oral epithelial IL-8 protein, hβD-2 mRNA and IL-8 mRNA. The first arrow represents a MOI of 1 bacterium per epithelial cell, the second an MOI of 10:1 and the third an MOI of 100:1.

↑ = an increase in protein production or mRNA expression compared to the control

↔ = the same level of protein production or mRNA expression compared to the control

↓ = a decrease in protein production or mRNA expression compared to the control

6.5 Discussion

There is substantial support for the hypothesis that bacterial components can stimulate h β D-2 transcription in human cells. However, no studies have yet demonstrated the effects of whole oral bacteria on oral epithelial cells. Results presented in previous chapters show that h β D-2 protein is expressed in oral epithelial tissue and that h β D-2 mRNA is induced in oral epithelial cells after stimulation with bacterial products.

Normal oral epithelia are continually exposed to commensal and potentially pathogenic organisms. In order to establish the possible functional role of beta-defensins in the oral cavity, this study has determined whether viable or heat-killed oral commensal and potentially pathogenic organisms are capable of regulating the production of the soluble inflammatory mediators IL-8 and h β D-2 by human oral epithelial cells.

The studies presented reveal that the oral commensals *S. gordonii* and *S. sanguis*, when incubated live with oral epithelial cells induce a decrease in h β D-2 mRNA, particularly at high MOIs. Interestingly, a similar effect is seen with the production of IL-8 protein from oral epithelial cells. Live *S. gordonii* at the highest MOI, leads to a decrease in IL-8 production. *S. sanguis* has a more marked effect, with greater decreases in IL-8 production at all MOIs. However, *S. gordonii* and *S. sanguis*, when heat-killed and incubated with oral epithelial cells, have different effects on oral epithelial h β D-2 mRNA levels. *S. gordonii* has no effect and *S. sanguis*, in contrast, stimulates increased expression of h β D-2 mRNA in oral epithelial cells.

The oral pathogen *P. gingivalis* stimulated increased expression of h β D-2 mRNA in oral epithelial cells at all MOIs and regardless of whether the bacteria were live or heat-killed. Whilst live *P. gingivalis* also upregulated the production of IL-8 protein from oral epithelial cells, this only occurred at the lowest MOI. Higher concentrations of live

P. gingivalis had no effect on oral epithelial IL-8 production. When exposed to heat-killed *P. gingivalis*, oral epithelial cells responded to the bacteria with the increased secretion of IL-8 protein. Results show that *P. gingivalis* had no effect on IL-8 mRNA in oral epithelial cells.

Both strains of *A. actinomycetemcomitans*, whether live or heat-killed, upregulated the production of IL-8 protein and hβD-2 mRNA from oral epithelial cells at all MOIs. The expression of mRNA for IL-8 differed, after incubation with live and heat-killed *A. actinomycetemcomitans* all concentrations of bacteria elicited a slight decrease in IL-8 mRNA expression in oral epithelial cells.

The effect of different microorganisms on human epithelial cell hβD-2 mRNA has been investigated by other groups. One of the first studies demonstrated that contact with heat-killed *P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus* and *Candida albicans* induced hβD-2 mRNA in foreskin-derived keratinocytes (Harder *et al.*, 1997). Subsequent studies investigated the effects of oral bacteria on oral epithelial cells with respect to their hβD-2 mRNA production. Krisanaprakornkit *et al.*, 2000, showed that hβD-2 mRNA is induced by a cell wall extract of *F. nucleatum*, an oral commensal, but not by that of *P. gingivalis*, a periodontal pathogen. Krisanaprakornkit *et al.*, 2000, suggest that commensal organisms have a role in stimulating mucosal epithelial cells and therefore maintaining the barrier that contributes to homeostasis and host defense.

Results presented herein show that the oral commensals *S. gordonii* and *S. sanguis* also upregulate the production of hβD-2 mRNA from oral epithelial cells. However, when exposed to greater numbers of live bacteria, oral epithelial cells showed the same or lower levels of hβD-2 mRNA compared to control cells. Also, when *S. gordonii* were heat-killed they no longer demonstrate any effect on OEC with regard to hβD-2 mRNA production. This suggests that *S. gordonii* needs to be metabolically active to elicit a stimulatory effect

on OECs. It may also suggest that the *S. gordonii* factor which stimulates the expression of hβD-2 mRNA in OECs may be heat labile.

In contrast, heat-killing of *S. sanguis* led to a greater stimulatory effect of the bacteria on production of hβD-2 mRNA from OECs. This suggests that an element of live *S. sanguis*, subsequently knocked out by heat inactivation, could be inhibiting the production of hβD-2 mRNA from OECs. It could also be suggested that *S. sanguis* downregulates hβD-2 mRNA and therefore any subsequent protein production because it is more susceptible to killing by hβD-2. There is no published evidence, to date, on the antimicrobial effects of hβD-2 on *S. sanguis* or *S. gordonii*.

In contrast to results shown by Krisanaprakornkit *et al.*, 2000 using *P. gingivalis* cell wall, results presented in this chapter show that whole *P. gingivalis* whether live or heat-killed stimulates the production of hβD-2 mRNA from OECs. Krisanaprakornkit *et al.*, (2000) suggest that the absence of hβD-2 mRNA induction by the *P. gingivalis* cell wall is consistent with the ability of this organism to evade this aspect of the host innate immune response, while *F. nucleatum* may help keep gingival epithelial cells in a stimulated state for effective and continuous host defense. A later paper published by this research group contradicted this hypothesis and these workers state that in contrast to *F. nucleatum*, only whole cells (not a cell wall extract) of *P. gingivalis* stimulate hβD-2 mRNA expression in oral keratinocytes (Dale and Krisanaprakornkit, 2001).

Upregulation of hβD-2 mRNA by *P. gingivalis* may be due to LPS. The activity of LPS has been shown to persist after heating (Bjornson *et al.*, 1988) and heat-inactivation of bacteria does not knock out the stimulatory effects of *P. gingivalis*. Previous results in Chapter 5 show that *P. gingivalis* LPS upregulates hβD-2 mRNA in OECs and also, as with whole bacteria, has no effect on the IL-8 protein and mRNA production from OECs.

Studies conducted in this chapter have also demonstrated the effect of another oral pathogen, *A. actinomycetemcomitans*, on oral epithelial cell hβD-2 mRNA production. Both an invasive and a non-invasive strain of *A. actinomycetemcomitans* stimulated increased expression of hβD-2 mRNA in OECs. This is the first reported incidence of any oral pathogen stimulating the increased expression of hβD-2 mRNA in oral cells. Stimulation of OEC by *A. actinomycetemcomitans* to express higher levels of hβD-2 mRNA may be due in part to the surface associated material of the organism. As demonstrated in Chapter 5, section 5.4.1.2, *A. actinomycetemcomitans* SAM stimulated increased expression of hβD-2 mRNA in OECs. Studies have demonstrated, however, that hβD-2 mRNA is still induced in OECs after the bacteria have been heat-killed. It has been shown that the component of *A. actinomycetemcomitans* SAM which stimulates the release of IL-6 from human fibroblasts is sensitive to both heat and trypsin treatment (Reddi *et al.*, 1996). If this were also the case for hβD-2 in oral epithelial cells, this suggests multiple stimulants are responsible for the increase in hβD-2 mRNA, one of which would include *A. actinomycetemcomitans* SAM.

HaCaT keratinocytes incubated with adherent *S. pyogenes*, but not non-adherent *S. pyogenes*, show increased expression of IL-1α, IL-1β and IL-8 (Wang *et al.*, 1997). *A. actinomycetemcomitans* and *P. gingivalis* can adhere to, and invade, oral epithelial cells (Meyer *et al.*, 1991, Duncan *et al.*, 1993, Sandros *et al.*, 1993, Meyer *et al.*, 1996, Rudney *et al.*, 2001). The invasive and binding capacities of *A. actinomycetemcomitans* are suggested to contribute to the enhanced cytokine induction in oral epithelial cells (Huang *et al.*, 1998). There appears to be greater expression of hβD-2 mRNA in oral epithelial cells stimulated with the invasive strain of *A. actinomycetemcomitans* SUNY 465, compared to the non-invasive SUNY 462 strain (chapter 7, figure 7.17), however results

are not quantitative. It would be interesting to investigate further whether hβD-2 mRNA induction is affected by the invasive and adhesion properties of oral bacteria.

A recent study by Dinulos *et al.*, 2003 has examined the hβD-2 mRNA stimulating activities of a number of skin bacteria. Using a model of bacterial skin infection, they tested the hypothesis that if hβD-2 plays a role, *in vivo*, in host defence against skin infections, especially against the common skin pathogens *S. pyogenes* and *S. aureus*, keratinocyte expression of hβD-2 mRNA would be upregulated in response to challenge with the live pathogenic bacteria. Also they hypothesised that if hβD-2 is active in innate defence, it would display potent antimicrobial activity against skin pathogens such as *S. pyogenes*, but not against a commensal organism. They found that expression of hβD-2 mRNA was consistently induced by those bacteria rarely implicated in skin disease, including *S. epidermidis*, *P. aeruginosa* and *E. coli*. They also showed that hβD-2 mRNA expression was variably induced by challenge with *S. aureus* (consistent inducer) and *S. pyogenes* (poor inducer), suggesting that hβD-2 expression may not be targeted directly towards cutaneous host defense against major skin pathogens per se (Dinulos *et al.*, 2003). There was no difference between live and heat-killed organisms (*S. pyogenes*, *P. aeruginosa*, *S. aureus* and *E. coli*) in their ability to induce antimicrobial peptide expression. This suggests that active bacterial growth or metabolism is not necessary to induce hβD-2 responses and that heat-killed bacterial preparations contained the component(s) responsible for triggering hβD-2 signalling, as reported for induction of hβD-2 expression upon bacterial challenge of oral mucosal epithelium (Krisanaprakornkit, 2000). Results presented in this chapter are consistent with this study. It has been shown that hβD-2 mRNA is induced in OECs by both oral pathogens and oral commensal

bacteria, and that heat-killed bacteria can elicit the same effect as live bacteria on OEC production of hβD-2 mRNA.

Oral viridans streptococci are potent stimulators of IL-8 production from the oral epithelial cell line, KB (Vernier *et al.*, 1996). Results in this chapter, in contrast to those of Vernier *et al.*, 1996 show that at the highest MOI investigated (100:1), *S. gordonii* DL1 Challis stimulated a decrease in IL-8 protein production from OEC compared to the control. This was also the case when *S. sanguis* were incubated with OECs. With both bacteria, when heat-killed, there was no effect on OEC IL-8 production. As with hβD-2 mRNA, this suggests that a bacterial metabolic effect is responsible for the action on OECs. *S. gordonii* had no effect on the IL-8 mRNA production from OECs, whereas *S. sanguis*, when live and heat-killed, upregulates IL-8 mRNA in OECs after 5h. This is in agreement with results presented by Krisanaprakornkit *et al.*, 2000 on the oral commensal *F. nucleatum*, which upregulates both hβD-2 mRNA and IL-8 mRNA after a 24h incubation period.

After 5h incubation with higher numbers of *P. gingivalis*, there was no difference in IL-8 secretion between stimulated and unstimulated control oral epithelial cells.

A report by Darveau *et al.*, 1998 suggests a novel pathogenic mechanism for *P. gingivalis*, namely, local chemokine paralysis. In contrast to the response elicited by other periodontal bacteria, gingival epithelial cells did not secrete IL-8 when coincubated with several different strains of the periopathogen *P. gingivalis*. This is consistent with the failure of *P. gingivalis* to elicit IL-8 accumulation from human endothelial cells (Darveau *et al.*, 1998). The lack of host cell detection of *P. gingivalis* has been proposed to contribute to bacterial colonisation of the host (Reife *et al.*, 1995). Darveau *et al.*, 1998, also demonstrated, more significantly, that *P. gingivalis* was able to inhibit IL-8 accumulation

induced by other bacteria. This could be due to *P. gingivalis* proteases, previously *P. gingivalis* proteases have been shown to degrade IL-1β and IL-6 (Fletcher *et al.*, 1997).

The induction of IL-8 and MCP-1 in human endothelial cells by live *P. gingivalis* was relatively weak compared with that by inactivated bacteria (Mao, 2002). This is in agreement with results presented in this chapter, particularly at higher MOI.

Similarly, both killed and viable *P. gingivalis* elicited the production of prostaglandin E2, interleukin-1 beta (IL-1 beta), IL-6 and IL-8, although killed *P. gingivalis* induced generally higher levels, particularly of IL-6 and IL-8, compared with the viable bacteria (Steffen *et al.*, 2000). The authors suggest that these findings would be predicted, since bacterial surface structures and components are directly related to the characteristics of bacterial interaction with, and triggering of intracellular signalling mechanisms in, the gingival fibroblast (Bliska cell 1993). Alternatively, activities of *P. gingivalis* macromolecules (that is proteases, outer membrane proteins), that could either degrade or bind host molecules from the cellular milieu, could explain these differences, since it could be expected that, in killing the bacteria, enzymatic functions were destroyed and outer membrane structures may have been modified. With either alternative, the results suggested that the viability and surface characteristics of *P. gingivalis* that interact with fibroblasts may initiate very different host cell responses.

Results have shown that *A. actinomycetemcomitans* induces the production of both IL-8 protein and hβD-2 mRNA. However, in oral epithelial cells both live strains of *A. actinomycetemcomitans* induced a decrease in the level of IL-8 mRNA detected.

Krisanaprakornkit *et al.*, 2000 have shown that IL-8 and hβD-2 mRNA are differentially regulated by an *F. nucleatum* cell wall extract in oral epithelial cells. They show that IL-8 is induced much more rapidly than hβD-2 mRNA, but hβD-2 mRNA is induced to a much

higher level compared to IL-8 mRNA. Results presented in this chapter suggest that IL-8 and h β D-2 mRNA may be differentially regulated.

In the case of *A. actinomycetemcomitans*, the pattern of h β D-2 mRNA expression parallels the release of IL-8 protein from OECs. Perhaps, as in the paper by Krisanaprakornkit *et al.*, 2000, IL-8 mRNA induction occurs more rapidly than h β D-2 mRNA. By the time a change is detected in the expression of h β D-2 mRNA the transcription of IL-8 mRNA has led to an increase in IL-8 protein which is comparable to h β D-2 mRNA.

It may be possible that bacterial adhesins play a role in the induction of inflammatory cytokines and host defense peptides in the oral cavity.

Rieder *et al.*, 1997 treated *H. pylori* with sodium azide rather than by heat-treatment; this killed the bacteria by inactivating their metabolism but did not lyse the bacterial cells, so that adherence still occurred. In contrast, boiling the bacteria to kill them or treating them with proteinase K would lead to damage of the cell wall and to loss of adhesins. The results of their stimulation assay indicate that there is an equally strong induction of IL-8 secretion in AGS cells after stimulation with live or metabolically inactive bacteria. Further results showed that not only live bacteria, but also metabolically inactive bacteria, increased IL-8 secretion, suggesting that the mechanism leading to IL-8 stimulation does not apparently rely on live bacteria and an active metabolism but needs an intact membrane conformation whereby the proteins on the bacterial surface are presented in a particular aggregation state.

Chapter 7

**The relationship between bacterial binding to and/or
invasion of oral epithelial cells and chemokine and
antibiotic peptide production**

Chapter 7

The relationship between bacterial binding to and/or invasion of oral epithelial cells and chemokine and antibiotic peptide production

7.1 Introduction

Interaction between bacteria and epithelia is critical in bacterial infections. Most obviously, adherence of bacteria to epithelial cells is required for colonisation (Finlay and Cossart 1997, Meyer *et al.*, 1997). Invasion of host epithelial cells is an important step in the pathogenesis of many diseases and may prove to be an effective way in which bacteria evade the host immune system. It has also been suggested that the binding and/or invasion of bacteria could contribute to the enhanced cytokine induction seen in oral epithelial cells after contact with pathogenic bacteria (Huang *et al.*, 1998).

As shown in Chapter 6, h β D-2 mRNA is induced in oral epithelial cells by both oral commensal and oral pathogenic bacteria. The oral bacteria used included both invasive and non-invasive strains. Previous studies have shown that in intestinal and skin environments, both invasive and non-invasive members of the microbiota stimulate increased expression of h β D-2 mRNA (O'Neil *et al.*, 1999, 2000, Dinulos *et al.*, 2003). To date, there is no literature which documents the effects that less invasive or adhesive oral bacteria may have on the h β D-2 mRNA expression in cells of the oral cavity.

The periodontal diseases represent a complex interaction of multiple organisms with host cells and tissues and it is therefore essential to look at the interactions between different periodontal bacteria and epithelial cells along with the immune responses involved. Using a human oral squamous carcinoma cell line the attachment and invasive properties of *S. gordonii*, *S. gordonii* isogenic mutants and two clinical isolates of *A. actinomycetemcomitans* were examined. Their role in the production of IL-8 and h β D-2 was also established.

7.2 Aims

To determine the effect of adhesion and invasion of oral bacteria on the production of human-beta defensins from oral epithelial cells.

7.3 Materials and methods

Invasion and adherence assays were carried as described in Chapter 2, section 2.11. All *S. gordonii* strains used in this chapter are described in Chapter 2, section 2.1.1. The possible role of the integrin receptor $\alpha 5\beta 1$ in *S. gordonii* adhesion to, and invasion of, oral epithelial cells was also determined. Prior to the addition of *S. gordonii*, oral epithelial cells were washed with PBS and fresh medium containing 2 μ g/ml anti- $\alpha 5\beta 1$ integrin antibody (clone JBS5, Chemicon, UK) was added. Cells were then incubated for 1 hour prior to addition of *S. gordonii* at a MOI of 100:1, the antibody remained present in the medium. The invasion and adherence assay was then carried out as described in Chapter 2, section 2.11.

To determine the effect of *S. gordonii* DL1 Challis, *S. gordonii* CshAB-, *S. gordonii* SspAB-, *S. gordonii* CshAB-/SspAB- (quad mutant), *S. gordonii* CshAB-/SspAB-/FbpA- (quint mutant), *A. actinomycetemcomitans* SUNY 462 and *A. actinomycetemcomitans* SUNY 465 on oral epithelial cell IL-8 and h β D-2 production, cells were incubated with bacteria as described in Chapter 2, section 2.10, for two hours. After incubation, cell supernatant was removed and analysed by ELISA to determine the IL-8 secretion from oral epithelial cells, as described in Chapter 2, section 2.3. Cell monolayers were washed twice with PBS and total cellular RNA extracted as described in section 2.4 for cells. Samples were then reverse transcribed, as described in section 2.5. 26 cycles of PCR were performed for GAPDH and 35 cycles for h β D-2 and IL-8, primers and programs were as

described in Table 2. 20µl of PCR product was loaded and run on a 2% agarose gel as described in Chapter 2, section 2.7.

7.4 Results

7.4.1 *S. gordonii* adhesion to, and invasion of, oral epithelial cells

7.4.1.1 *S. gordonii* DL-1 Challis SspAB- mutant strain

To determine the role of SspAB in the binding and/or invasion of *S. gordonii*, the wild type or the SspAB- mutant was co-cultured with oral epithelial cells and the binding and/or invasion assessed.

As shown in Fig. 7.1 only the lowest MOI revealed a difference in binding of the wild type and mutant organism. At the medium and highest MOI the difference in adherence was not significant. There was no invasion at the lowest MOI and no significant difference in uptake at higher bacteria:epithelial cell ratios.

All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

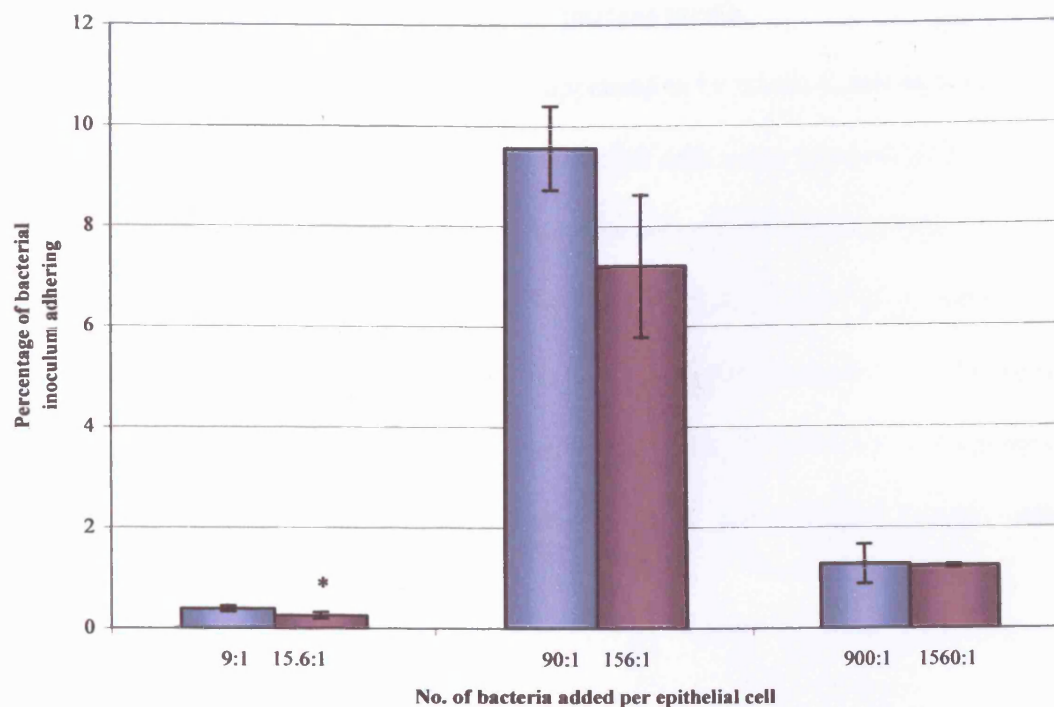


Figure 7.1

Adhesion of *S. gordonii* SspAB- mutant to oral epithelial cells■ *S. gordonii* ■ *S. gordonii* SspAB- mutant

Results shown are the mean of triplicate samples and represent one experiment

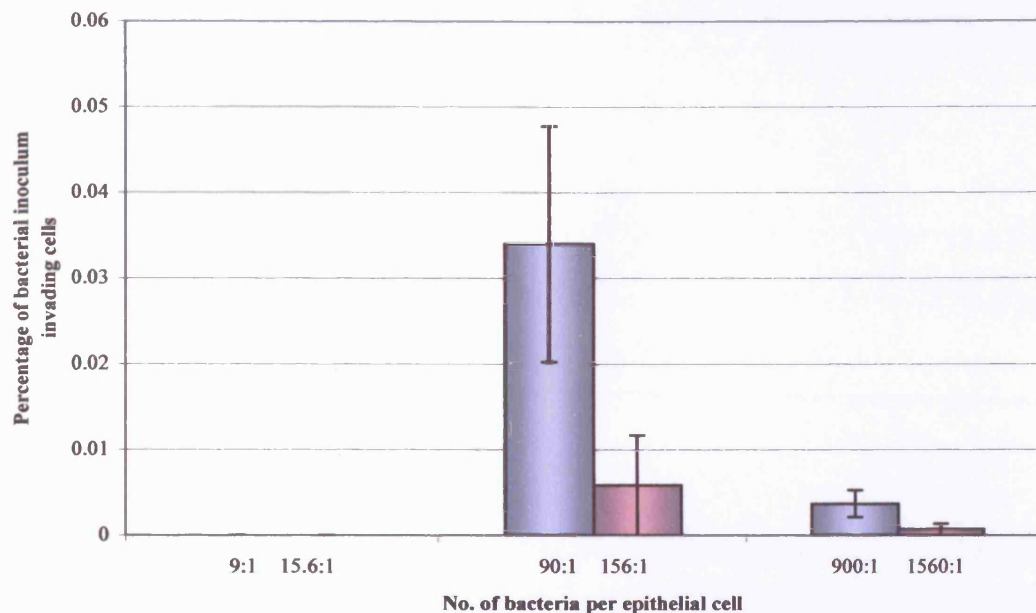
* $p < 0.05$ 

Figure 7.2

Invasion of oral epithelial cells by *S. gordonii* SspAB- mutant■ *S. gordonii* ■ *S. gordonii* SspAB- mutant

Results shown are the mean of triplicate samples and represent one experiment

7.4.1.2 *S. gordonii* DL-1 Challis CshAB- mutant strain

The role of SspA or B in binding/invasion appeared to be minimal and so it was decided to examine the role of CshA or CshB. Oral epithelial cells were exposed to *S. gordonii* DL1 Challis or the mutant organism lacking CshAB as described in the methods.

Testing the binding and invasion of the wild type and CshAB- mutant at these MOIs (low, medium and high) revealed that CshAB plays a significant role in adherence at the intermediate MOI but this cell surface protein appeared to play no role in invasion (Fig. 7.3 and 7.4) All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

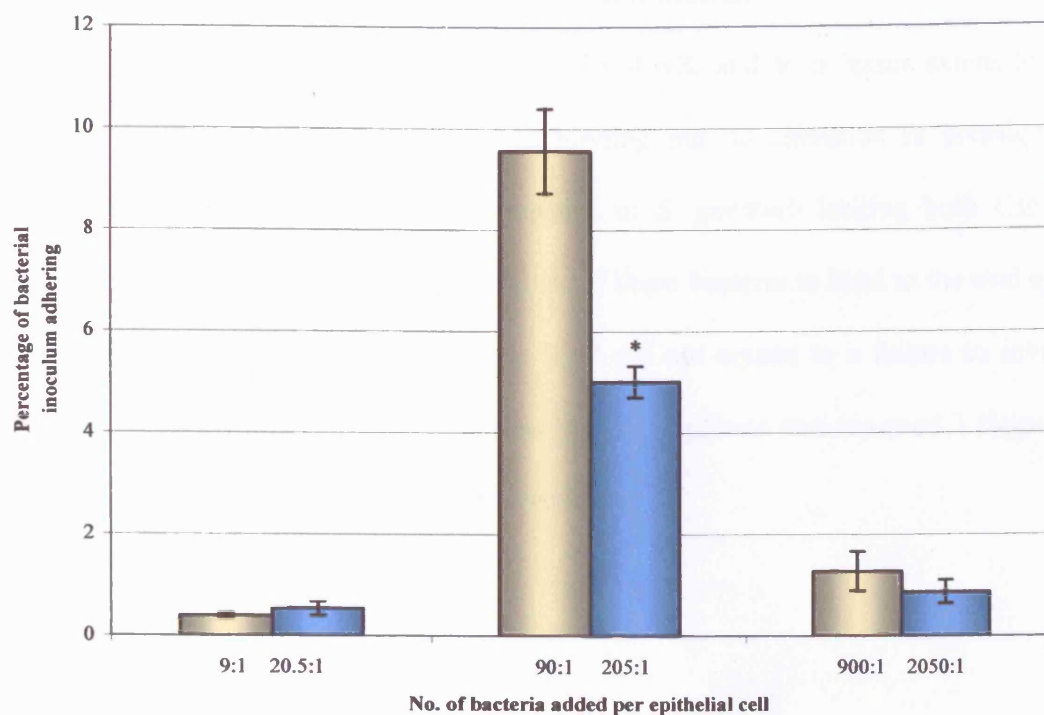


Figure 7.3 Adhesion of *S. gordonii* CshAB- mutant to oral epithelial cells
 ■ *S. gordonii* ■ *S. gordonii* CshAB- mutant
 Results shown are the mean of triplicate samples and represent one experiment * $p < 0.05$

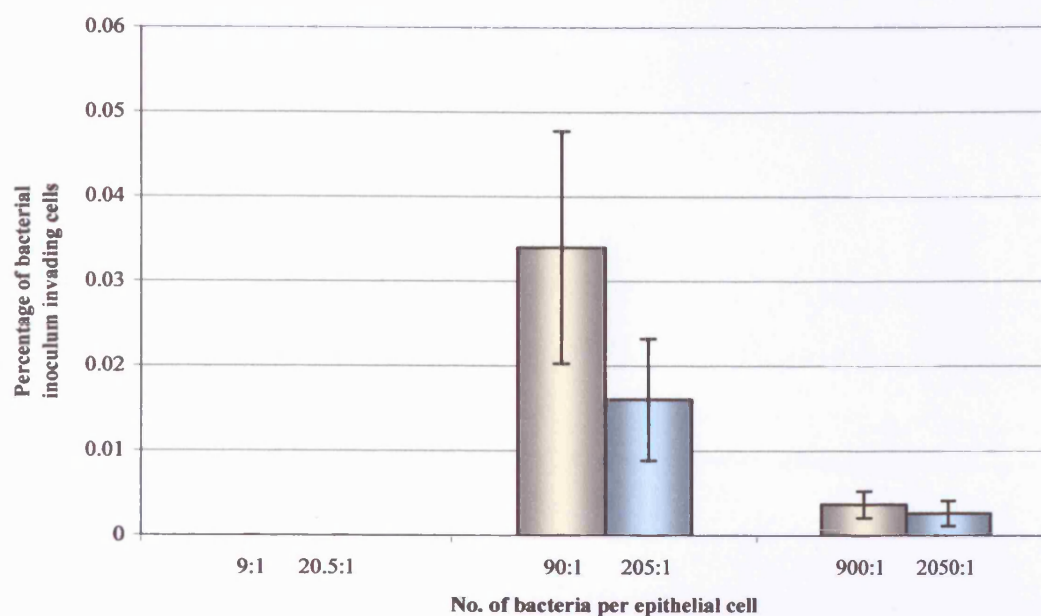


Figure 7.4 Invasion of oral epithelial cells by *S. gordonii* CshAB- mutant
 ■ *S. gordonii* ■ *S. gordonii* CshAB- mutant
 Results shown are the mean of triplicate samples, and represent one experiment.

7.4.1.3 Adhesion to, and invasion of, oral epithelial cells by *S. gordonii* DL-1 Challis quad and *S. gordonii* DL-1 Challis quint mutants

The above results have revealed that loss of CshAB, and to a lesser extent SspAB, is associated with a significant decrease in binding but no alteration in invasion. When epithelial cells were cultured in the presence of *S. gordonii* lacking both CshAB and SspAB, there was an almost complete failure of these bacteria to bind to the oral epithelial cells. Perhaps surprisingly, this failure to bind did not equate to a failure to invade (see Figures 7.5 and 7.6). The assay was carried out in triplicate and repeated 3 times; results shown are representative of triplicate experiments.

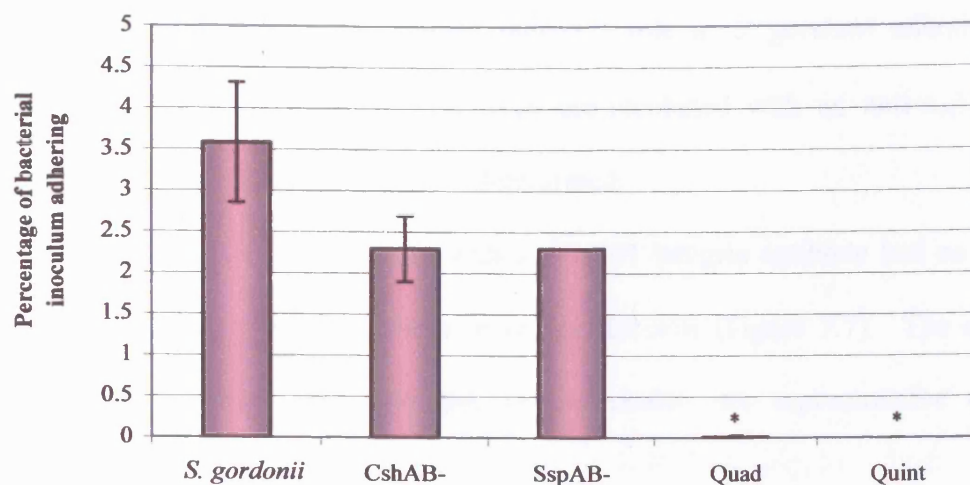


Figure 7.5 Adhesion of *S. gordonii* CshAB-, *S. gordonii* SspAB-, *S. gordonii* quad and *S. gordonii* quint mutants to oral epithelial cells, compared to *S. gordonii* DL1 Challis. Results show the mean of triplicate samples, and are representative of triplicate experiments
* $p < 0.05$

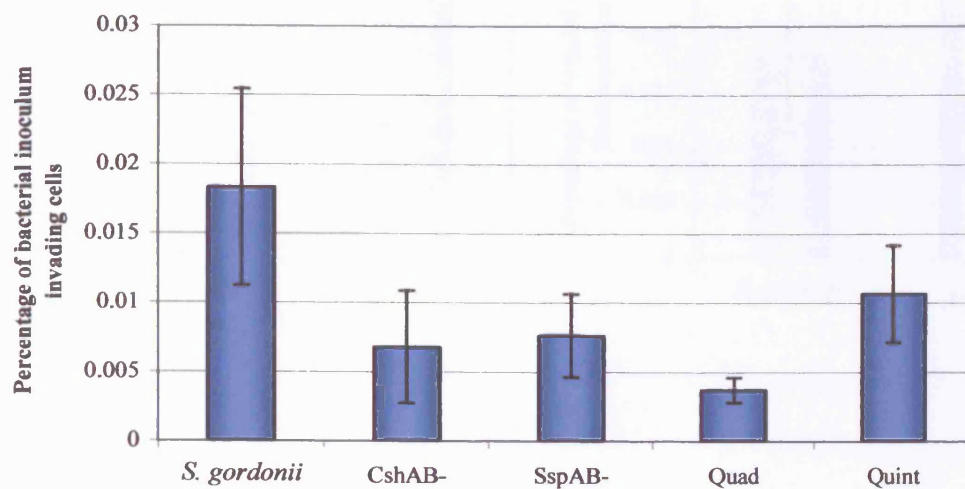


Figure 7.6 Invasion of oral epithelial cells by *S. gordonii* CshAB-, *S. gordonii* SspAB-, *S. gordonii* quad and *S. gordonii* quint mutants, compared to *S. gordonii* DL1 Challis. Results show the mean of triplicate samples, and are representative of triplicate experiments.

7.4.1.4 The effect of anti- $\alpha_5\beta_1$ -integrin on adhesion to and invasion of oral epithelial cells by *S. gordonii*

To determine whether the $\alpha_5\beta_1$ integrin played a role in *S. gordonii* adhesion to, or invasion of, oral epithelial cells, cells were pre-incubated with an anti- $\alpha_5\beta_1$ integrin antibody and adhesion and invasion were determined.

Pre-incubation of oral epithelial cells with anti- $\alpha_5\beta_1$ integrin antibody had no effect on the ability of this bacterium to adhere to or invade cells (Figure 7.7). The assay was carried out in triplicate and repeated; results shown are representative of repeat experiments.

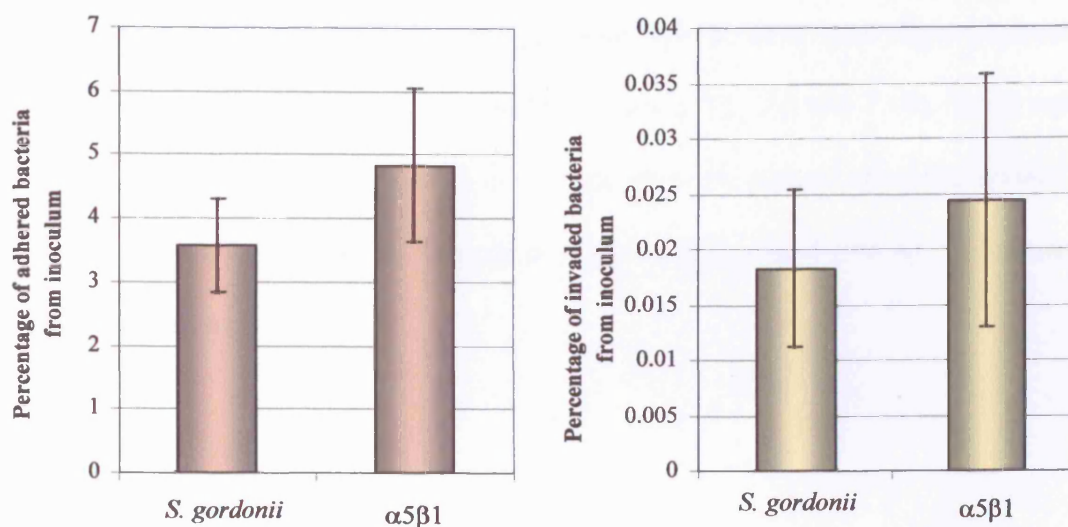


Figure 7.7 The effect of anti- $\alpha_5\beta_1$ integrin antibody on the adhesion to, and invasion of, oral epithelial cells by *S. gordonii* DL1 Challis. The graph on the left (orange) shows adhesion and the graph on the right (yellow) shows invasion.

7.4.2 The effect of *S. gordonii* adhesion/invasion on IL-8 protein production by oral epithelial cells

Oral epithelial cell monolayers were incubated with *S. gordonii* DL-1 Challis, *S. gordonii* CshAB- and *S. gordonii* SspAB- for two hours and IL-8 production assessed. Cell monolayers were also incubated with 1µg/ml *E. coli* LPS, since previous results demonstrated that *E. coli* LPS upregulates IL-8 and hβD-2 in oral epithelial cells (see Chapter 4, sections 4.3.1 and 4.3.2). Oral epithelial cells increased their production of IL-8 2-fold compared to the control after stimulation with 1µg/ml LPS for 2 hours. After incubation with all *S. gordonii* strains at the highest MOI, there were small decreases in the production of IL-8 from oral epithelial cells compared to the control (Figures 7.8, 7.9 and 7.10). Also at the two lower MOIs for all *S. gordonii* strains; there were slight increases in the production of IL-8 compared to the control, (Figures 7.8, 7.9 and 7.10). These results were found not to be statistically significant compared to the control using the student's t-test. All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

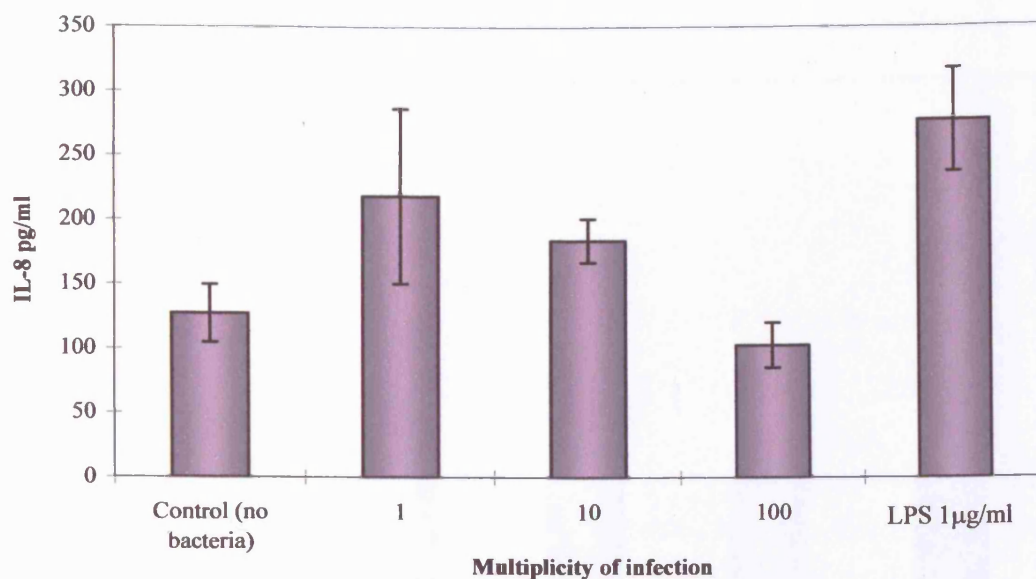


Figure 7.8 Production of IL-8 from oral SCC cell line H357 after 2 hour incubation with *S. gordonii* DL1 Challis. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk.

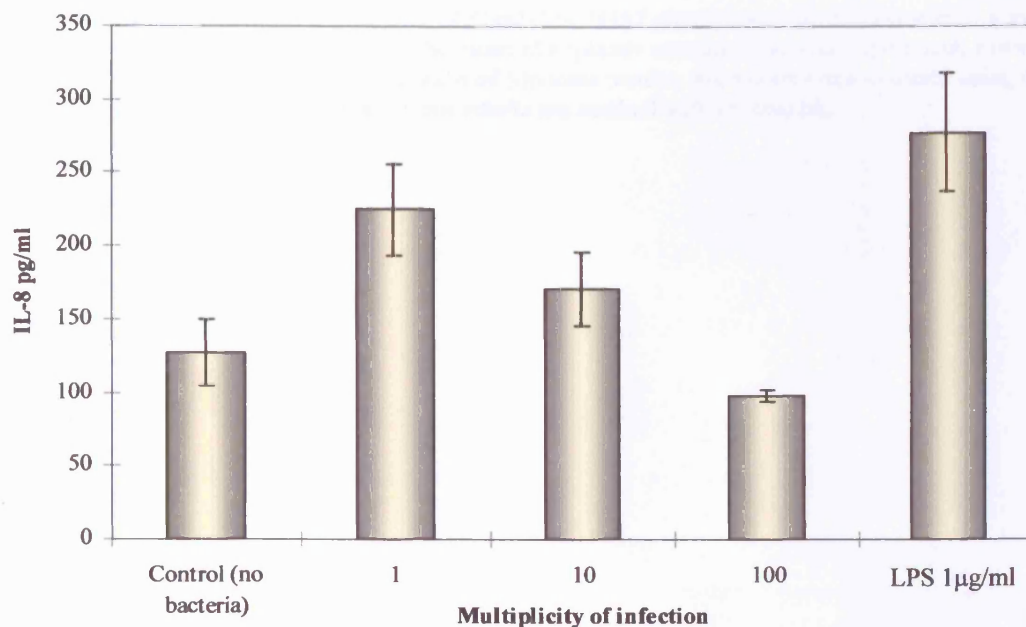


Figure 7.9 Production of IL-8 from oral SCC cell line H357 after 2 hours incubation with *S. gordonii* SspAB- mutant. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk.

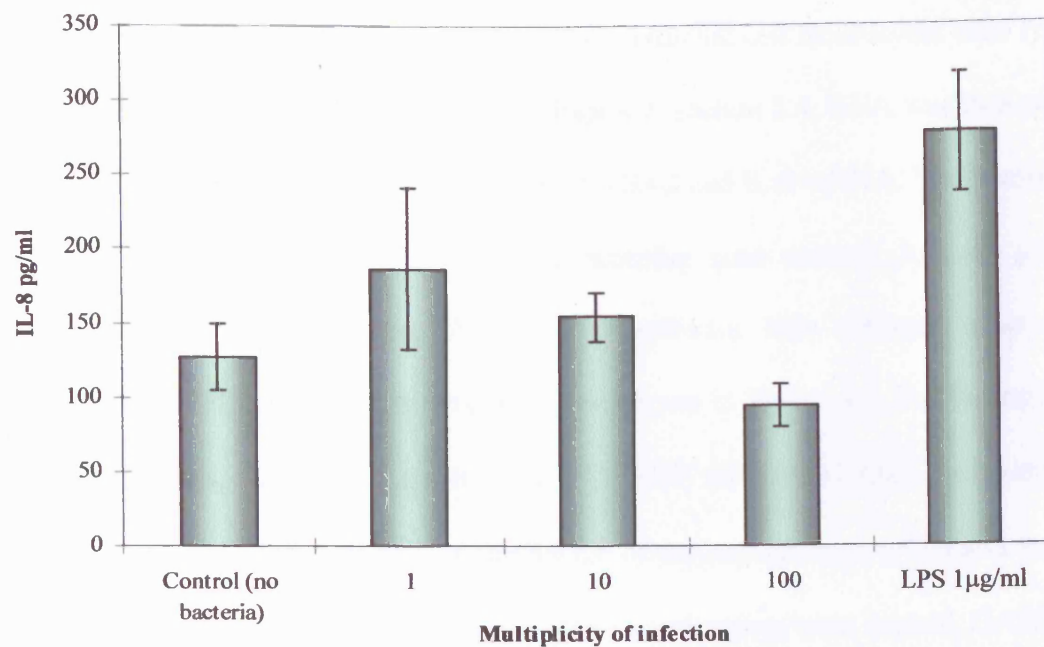


Figure 7.10 Production of IL-8 from oral SCC cell line H357 after 2 hours incubation with *S. gordonii* CshAB-. Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk.

7.4.3 Production of IL-8 and h β D-2 mRNA by oral epithelial cells after incubation with *S. gordonii* strains

After 2 hours co-culture with *S. gordonii* strains, epithelial cell monolayers were lysed and RNA extracted as described previously in Chapter 2, section 2.4. RNA was then subjected to RT-PCR in order to determine expression of h β D-2 and IL-8 mRNA. The housekeeping gene GAPDH was used as a positive housekeeping gene control. Agarose gels were analysed by densitometry using Phoretix 1D software, this software measures and compares the pixel intensity of bands like those shown in Figure 4.7. For the purposes of those experiments, bands were measured for GAPDH and h β D-2 from each sample and compared, h β D-2 is represented as a percentage of the respective GAPDH and the results expressed as arbitrary units. In all samples, the housekeeping gene control, GAPDH, was uniformly expressed by oral epithelial cells (Figure 7.11). IL-8 mRNA was present in all samples, and was upregulated; compared to the control, in oral epithelial cells by 1 μ g/ml LPS and all strains of *S. gordonii* at all MOIs. The expression of h β D-2 is upregulated by 1 μ g/ml LPS, as determined by densitometry (Figure 7.12). Oral epithelial cells incubated with *S. gordonii* at an MOI of 10:1 decreased expression of h β D-2 mRNA compared to control cells. After incubation with *S. gordonii* at MOIs of 1:1 and 100:1, there was no detectable h β D-2 mRNA, compared to control cells. When oral epithelial cells were exposed to *S. gordonii* CshAB- at MOIs of 10 and 100:1, h β D-2 mRNA levels were similar to control cells. In contrast, incubation with *S. gordonii* CshAB- at an MOI of 1:1 showed no detectable h β D-2 mRNA. When incubated with *S. gordonii* SspAB- at MOIs of 1:1 and 100:1, oral epithelial cells expressed mRNA for h β D-2 at levels similar to control cells. After incubation with *S. gordonii* SspAB- at an MOI of 10:1, there was no detectable h β D-2 mRNA. The expression of h β D-2 mRNA in oral epithelial cells in this figure (7.11) highlights some of the problems faced with the use of the PCR method. For example, incubation of oral epithelial cells with *S. gordonii* SspAB- shows mRNA similar to the

control at an MOI of 1:1, no expression at an MOI of 10:1, and h β D-2 mRNA levels similar to the control at an MOI of 100:1. Errors could have been amplified by these methods, for example the making of cDNA, some mRNA is less stable and so sample variation is more likely, also PCR reactions can vary too. All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

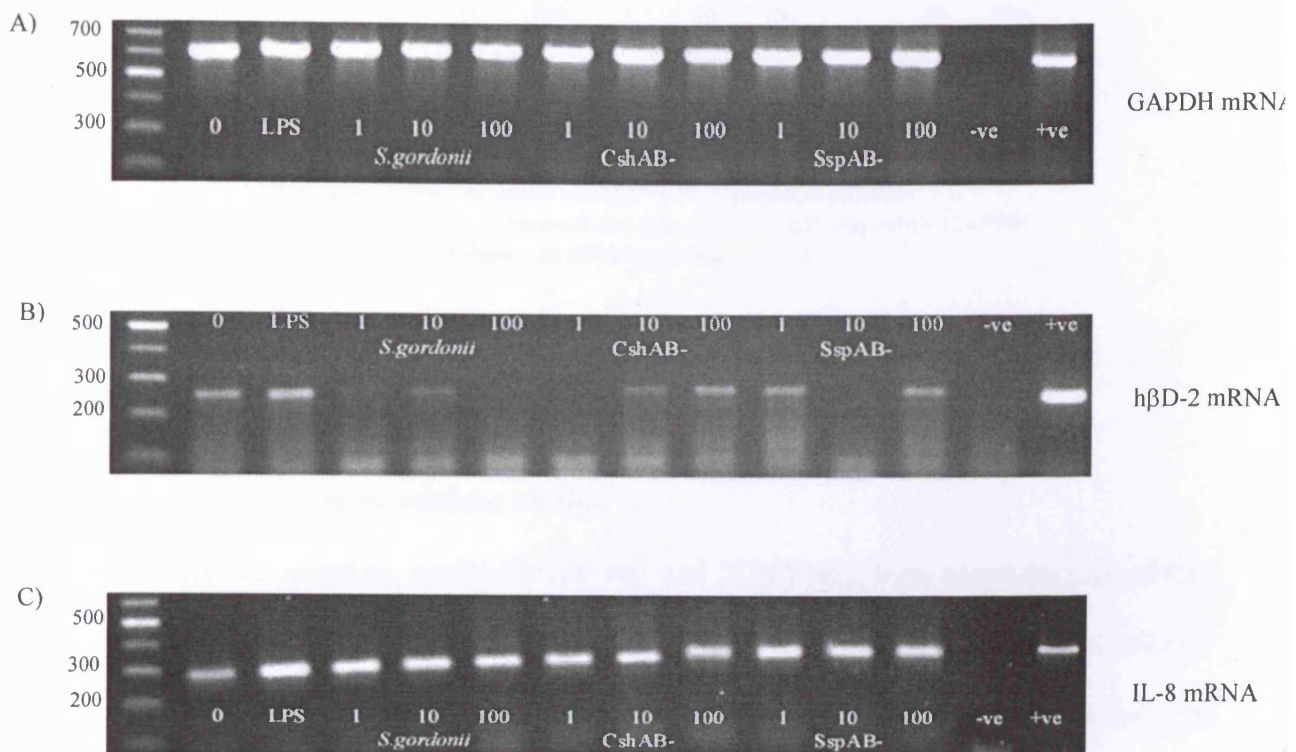


Figure 7.11 The expression of A) the housekeeping gene GAPDH, B) h β D-2 and C) IL-8 mRNA in oral epithelial cells after 2 hours incubation with 1 μ g/ml LPS, *S. gordonii*, *S. gordonii* CshAB- and *S. gordonii* SspAB-
 1 = MOI of 1:1
 10 = MOI of 10:1
 100 = MOI of 100:1

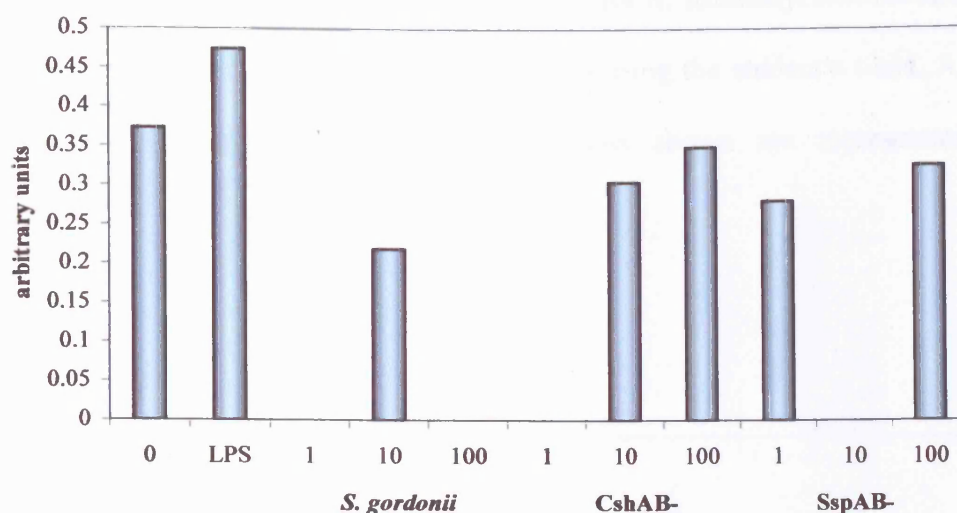


Figure 7.12 Densitometry analysis of bands for hβD-2 PCR products as seen in Figure 7.11. hβD-2 is expressed as a percentage of the respective GAPDH for each sample and is shown as arbitrary units.

7.4.4 Attachment to and invasion of oral epithelial cells by *A. actinomycetemcomitans* strains

A. actinomycetemcomitans strains SUNY 465 and SUNY 462 were tested for their ability to adhere to and invade epithelial cells using the oral SCC cell line H357. Strain SUNY 465 exhibited significantly greater levels of total associated bacteria to oral epithelial cells compared to strain *A. actinomycetemcomitans* SUNY 462 (Figure 7.10). At the highest MOIs, the percentage of the *A. actinomycetemcomitans* SUNY 465 bacterial inoculum associated with oral epithelial cells was significantly higher ($p < 0.005$) at 11.1%, compared to 1.26% of the *A. actinomycetemcomitans* SUNY 462 inoculum (Figure 7.13).

Strain *A. actinomycetemcomitans* SUNY 465 also exhibited significantly greater invasive properties (Figure 7.14). At the highest MOI, a significantly ($p < 0.05$) higher percentage, 0.3%, of the *A. actinomycetemcomitans* SUNY 465 inoculum had invaded oral epithelial cells, compared to 0.00067% of the *A. actinomycetemcomitans* SUNY 462 bacterial inoculum. This was also seen at lower MOIs, at an MOI of 15:1 for

A. actinomycetemcomitans SUNY 462 and 5:1 for *A. actinomycetemcomitans* SUNY 465 the result was found to be significant ($p < 0.05$) using the student's t-test. All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

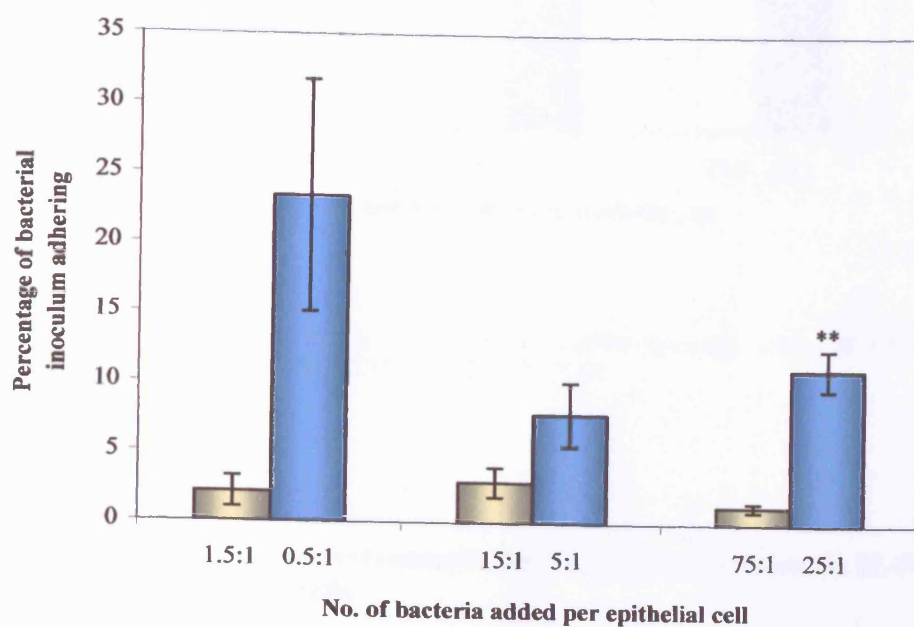


Figure 7.13

Adhesion of *A. actinomycetemcomitans* strains to oral epithelial cell
■ SUNY 462 ■ SUNY 465 ** $p < 0.005$

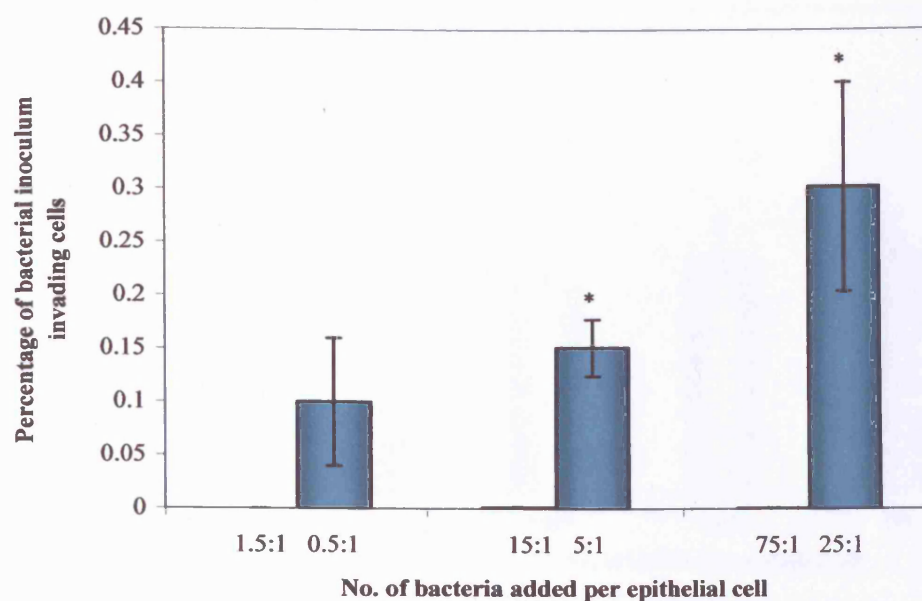


Figure 7.14

Invasion of oral epithelial cells by *A. actinomycetemcomitans* SUNY strains
 SUNY 462
 SUNY 465
 * $p < 0.05$

7.4.5 The effect of *A. actinomycetemcomitans* adhesion/invasion on IL-8 production from oral epithelial cells

The levels of IL-8 secreted by oral epithelial H357 cells after incubation with both *A. actinomycetemcomitans* SUNY strains were also measured. After 2h of infection, strain SUNY 465 upregulated the production of IL-8 protein. At an MOI of 180:1 there was a 2-fold increase in IL-8 production by oral epithelial cells (Figure 7.16). After incubation with SUNY 462 for the same period of time, oral epithelial cells showed a slight decrease in IL-8 production (Figure 7.15). Incubation with 1µg/ml LPS for 2h showed a slight increase in IL-8 production. However, after statistical analysis of the data for IL-8 production, none of the differences were found to be significant. All assays were carried out in triplicate and repeated; results shown are representative of these experiments.

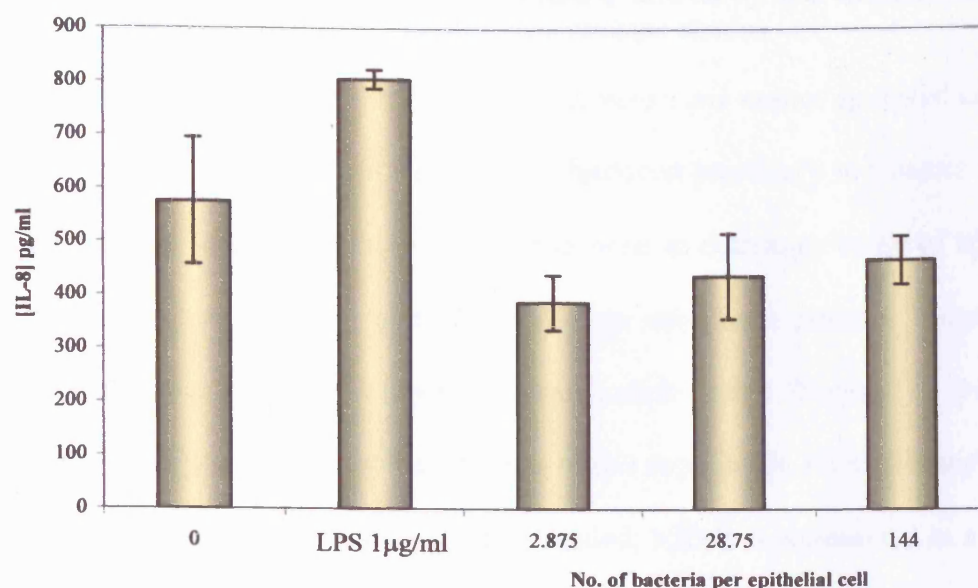


Figure 7.15 Production of IL-8 from oral SCC cell line H357 after 2h incubation with *A. actinomycetemcomitans* SUNY 462 Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk.

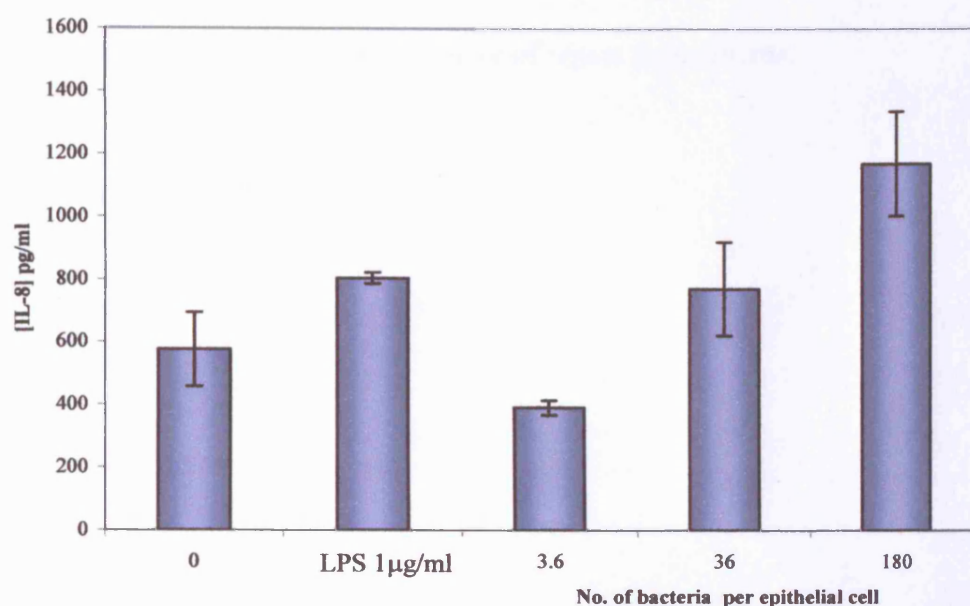


Figure 7.16 Production of IL-8 from oral SCC cell line H357 after 2h incubation with *A. actinomycetemcomitans* SUNY 465 Results shown are the mean of triplicate samples from one experiment. Error bars represent the standard deviation of triplicate results. All results were analysed using the Student's t-test and significant results are marked with an asterisk.

7.4.6 Production of interleukin-8 and hβD-2 mRNA by oral epithelial cells after incubation with *A. actinomycetemcomitans* strains

After 2 hours incubation with *A. actinomycetemcomitans* strains, epithelial cell monolayers were lysed and the RNA was extracted as described previously in Chapter 2, section 2.4. The RNA was then subjected to RT-PCR in order to determine levels of hβD-2 and IL-8 mRNA. The housekeeping gene GAPDH was used as a positive housekeeping gene control. Agarose gels were analysed by densitometry using Phoretix 1D software, results are shown in Figure 7.18. For the purposes of this experiment, for each sample, bands were measured for GAPDH and hβD-2 and compared; hβD-2 is represented as a percentage of the respective GAPDH and results expressed as arbitrary units. In all samples, the housekeeping gene control, GAPDH, showed the same level of expression. hβD-2 mRNA was expressed in all samples and was upregulated by 1µg/ml *E. coli* LPS and strains SUNY 465 and SUNY 462 at all MOIs (Figures 7.17, and 7.18). There was constitutive expression of IL-8 mRNA in all samples. All assays were carried out in triplicate and repeated; results shown are representative of repeat experiments.

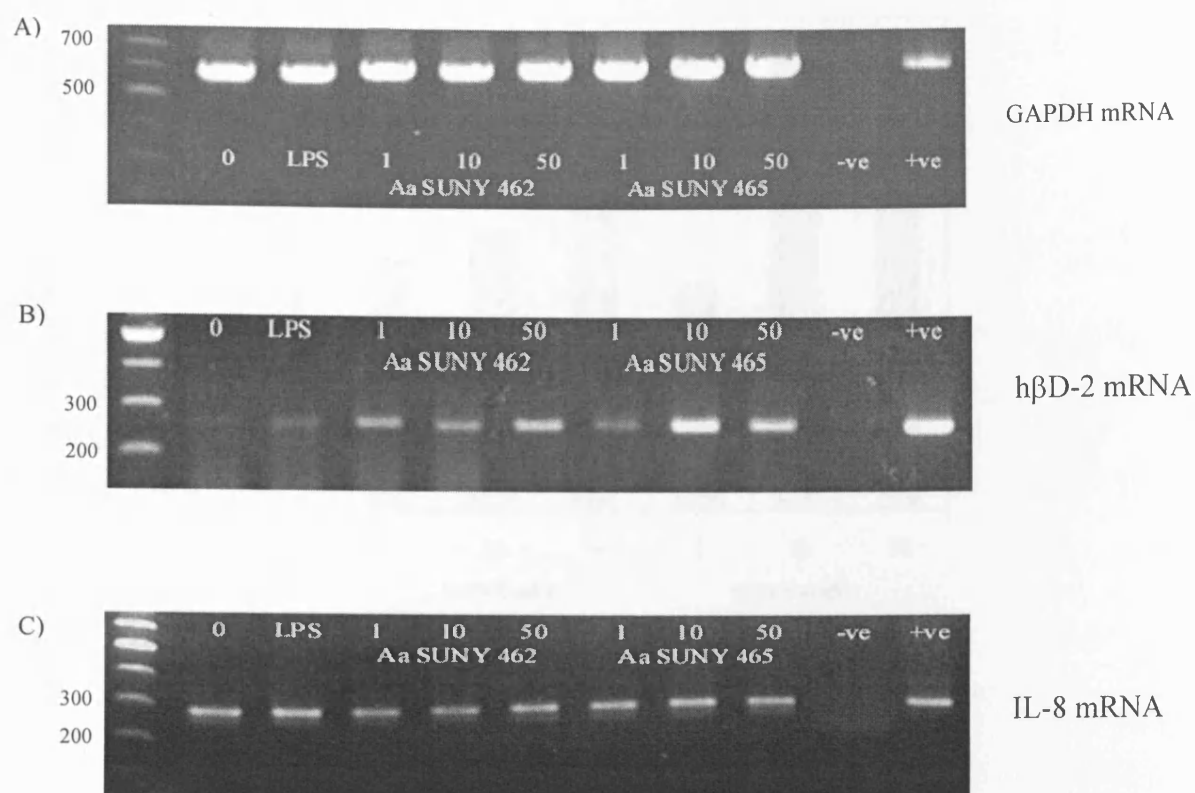


Figure 7.17 The relative expression of mRNA in oral epithelial cells after 2 hours incubation with *A. actinomycetemcomitans* SUNY 462 and *A. actinomycetemcomitans* SUNY 465. A) the housekeeping gene GAPDH, B) hβD-2 and c) IL-8 mRNA
 1 = MOI of 1:1
 10 = MOI 10:1
 50 = MOI of 50:1

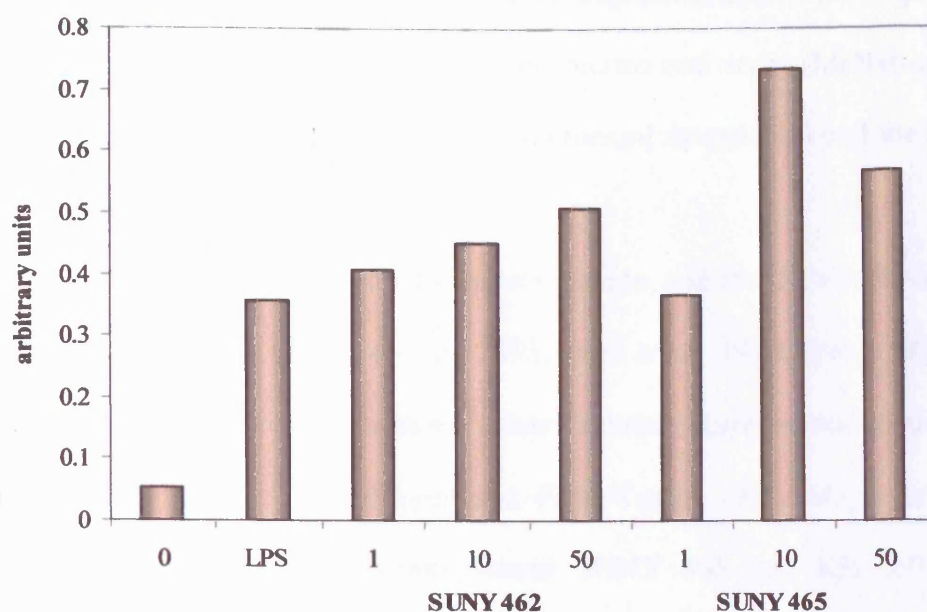


Figure 7.18 Densitometry analysis of bands for hβD-2 PCR products as seen in figure 7.14. hβD-2 is expressed as a percentage of the respective GAPDH for each sample and shown as arbitrary units.

7.5 Discussion

Adhesion of bacteria to epithelial cells can result in cellular, tissue and organismal pathology or it may merely be part of the complex, but perfectly normal, interactions between the host and its microbiota. The interactions of bacteria with epithelial cells may result in the binding of the bacteria to the cells with induction of defence proteins such as antibacterial peptides and cytokines. It may also lead to invasion with additional signals provoking, or not, defence processes. To study the influence of the initial adhesive event on cellular responses, this study has used two members of the normal oral microbiota: the Gram-positive *S. gordonii* (Whiley and Beighton, 1999) and the Gram-negative *A. actinomycetemcomitans* (Henderson *et al.*, 2003). *S. gordonii* is known to produce a number of complex high molecular mass adhesins such as CshA and CshB. Previous studies show that insertional mutations within *cshA*, and to a lesser extent *cshB* on *S. gordonii*, reduces both cell-surface hydrophobicity and the ability to adhere to the oral

bacterium *Actinomyces naeslundii*. However, expression of both polypeptides was found to be necessary for streptococci to colonise the murine oral cavity (McNab *et al.*, 1994). Little is known about the interactions of oral commensal streptococci and their surface proteins with epithelial cells.

Many oral pathogens have been shown to adhere to, and invade, a wide variety of oral cells (Duncan *et al.*, 1993, Sandros *et al.*, 1993, Dorn *et al.*, 1998, Han *et al.*, 2000). It is well documented that *A. actinomycetemcomitans* strains adhere to, and invade, oral epithelial cells (Meyer *et al.*, 1991, Meyer and Fives-Taylor 1994, Meyer *et al.*, 1996). The penetration of *A. actinomycetemcomitans* SUNY 465 into KB cells, a human oral epidermoid carcinoma cell line, has been demonstrated by a quantitative cell culture assay, with gentamicin used to kill external cells (Meyer *et al.*, 1991). Differences in invasiveness correlate with bacterial colonial morphology, smooth variants invade more proficiently than rough variants. Invasion of oral epithelial cells by *A. actinomycetemcomitans* is unlikely to be an artefact of cell culture since *A. actinomycetemcomitans* and *P. gingivalis* have been shown inside buccal mucosa cells taken *in vivo* (Rudney *et al.*, 2001).

The results of the studies presented reveal that isogenic mutants of the oral bacterium *S. gordonii* DL1 Challis, which are deficient in the production of SspA and SspB or CshA and CshB proteins showed reduced levels of total oral epithelial cell associated bacteria. The number of bacteria which had invaded oral epithelial cells was not significantly different to the controls.

Further to these observations, it was established that a *S. gordonii* DL1 Challis mutant strain lacking CshA, CshB, SspA and SspB (quad) cell surface proteins resulted in a further reduction in the number of bacteria that adhered to oral epithelial cells. The *S. gordonii* DL1 Challis quint mutant, which further lacks FbpA, a fibronectin binding protein, displayed similar adhesion properties to that of the quad mutant strain. However,

both strains invaded at levels comparable to the *S. gordonii* CshAB- and *S. gordonii* SspAB- mutants. These results suggest that antigen I/II family proteins SspA and SspB, also the cell wall proteins CshA and CshB are necessary for maximum *S. gordonii* adhesion to, and invasion, of the oral epithelial cell line H357.

Recent studies on endothelial cells have determined that the cell surface proteins CshA and CshB are required for maximum host cell invasion by *S. gordonii*. However, the deletion of genes for the antigen I/II adhesins, SspA and SspB, did not affect invasion (Stinson *et al.*, 2003).

Adhesins are bacterial components which mediate interactions between the bacterium and host cell surface. Bacterial engagement of host cell receptors can be a means of targeting a pathogen to a particular niche, co-opting underlying signalling pathways, establishing persistent infections and inducing invasion (Finlay and Cossart 1997). Invasion affords bacteria protection from immune detection and facilitates access to deeper tissues. The internalisation of *S. aureus* by endothelial cells, osteoblasts and 293 cells has been shown to require the host cell integrin $\alpha 5\beta 1$ (Massey *et al.*, 2001, Ahmed *et al.*, 2001 and Sinha *et al.*, 1999).

Oral epithelial cell line H357 expresses $\alpha 5\beta 1$ integrin on the cell surface, as determined using flow cytometry in Chapter 4, section 4.3.5. However, results presented herein show that the integrin $\alpha 5\beta 1$ played no role in the adhesion and invasion ability of *S. gordonii* with the oral epithelial cell line H357. H357 oral epithelial cells express a wide range of other integrins on their cell surface (Thomas *et al.*, 2001 and Sugiyama *et al.*, 1993) which may play a role in bacterial adhesion.

It has been shown that anti- $\alpha 5\beta 1$ integrin blocks the internalisation of *S. aureus* into oral epithelial cell lines H357 and H376 (Kintarak *et al.*, 2004). In freshly isolated oral keratinocytes there were mixed results where anti- $\alpha 5\beta 1$ integrin, in some instances,

blocked the internalisation of *S. aureus* and in some cases it failed to do so (Kintarak *et al.*, 2004).

P. gingivalis uses direct binding to the $\beta 1$ integrin, whilst *S. pyogenes* and *S. aureus* use fibronectin as a bridging molecule to bind to the integrin receptor (Sinha *et al.*, 1999, Molinari *et al.*, 2000, Yilmaz *et al.*, 2002).

A fibronectin-binding surface protein of *S. pyogenes*, SfbI protein, has been implicated in both adherence and invasion processes. In strain A40, a well characterised clinical isolate, which expresses SfbI, SfbI was the main factor required for attachment and invasion by using fibronectin as a bridging molecule and the $\alpha_5\beta_1$ integrin as the cellular receptor (Molinari *et al.*, 2000). It may be that in addition to the expression of $\alpha_5\beta_1$ integrin on the surface of H357 oral epithelial cells, the introduction of a bridging molecule, such as fibronectin may enhance the invasive ability of *S. gordonii*. Interestingly, studies have shown that the cell surface proteins CshA and CshB have been reported to be responsible for mediating streptococcal adhesion to immobilised fibronectin (McNab *et al.*, 1994).

An important event in determining the consequences of bacterial colonisation, which occurs early in the interactive process, is the induction of pro-inflammatory or anti-inflammatory cytokines and chemokines in response to bacteria. The wild type strain of *S. gordonii*, DL1 Challis, and the isogenic mutants, *S. gordonii* CshAB- and *S. gordonii* SspAB- had similar effects on the production of IL-8 (protein and mRNA) and h β D-2 mRNA from OECs. The reduced adhesion and invasion properties of the *S. gordonii* DL1 Challis mutant strains did not elicit a different cytokine and antimicrobial peptide response in oral epithelial cells, compared to the wild type strain.

Binding of the *S. mutans* Sr (antigen I/II) protein to the carbohydrate moieties of monocyte membrane glycoproteins elicits the production of TNF- α , IL-1 β and IL-6 (Soell *et al.*,

1994). Also, antigen I/II polypeptides and cell-surface rhamnose-glucose polymers (RGPs) may mediate the ability of oral streptococci (including mutans group streptococci, *S. anginosus*, *S. constellatus*, *S. gordonii*, *S. intermedius*, *S. milleri*, *S. oralis* and *S. salivarius*) to stimulate the production of IL-8 in oral epithelial cell line KB and of IL-8 and IL-6 in endothelial cells (Vernier *et al.*, 1996).

The study by Vernier *et al.*, (1996) demonstrated that the protein I/IIf and RGPs, two major adhesins from *S. mutans* OMZ 175, specifically bind to KB and endothelial cells in a dose-dependent, saturable fashion in the absence of serum. Furthermore, the binding of these molecules initiates the cellular response, which results in the release of IL-8 and IL-6 from endothelial cells. These data suggest that protein I/IIf and RGPs play a very important role in the interactions between bacteria and KB or endothelial cells insofar as similar cytokine profiles were obtained when cells were stimulated with whole bacteria or surface components. Moreover, as well as the pivotal role these adhesins play as cell surface components, they can be released from the cell surface and act at a distance with the potential capacity of accelerating the inflammatory process by stimulating monocytes, epithelial and endothelial cells (Vernier *et al.*, 1996)

The results presented show that whilst the antigen I/II proteins and the cell surface proteins CshA and CshB play a role in the adhesion of oral epithelial cells by *S. gordonii*, they do not appear to stimulate an increase in either IL-8 (protein and mRNA) or hβD-2 mRNA. Both the wild type strain *S. gordonii* DL1 Challis and mutants lacking CshA, CshB, SspA and SspB had similar effects on oral epithelial cell IL-8 and hβD-2 production/expression. However, after increased exposure to the wild type *S. gordonii*, as shown in Chapter 6, section 6.4.1.1 and 6.4.1.2 there is an increase in the production of IL-8 protein and hβD-2 mRNA at certain MOIs. It may be that these proteins are being released from the cell surface of *S. gordonii* and the increased exposure time is sufficient for enough protein to accumulate and an inflammatory response to be seen.

It has been suggested that at least two streptococcal adhesins, LTA and M protein, are involved in the adherence of streptococci to certain cells and that the relative contributions of these adhesins to the attachment process depends on the type of host cells used to study adherence (Courtney *et al.*, 1992). Interestingly the present studies have shown that LTA from *S. sanguis* stimulates oral epithelial cells to produce hβD-2 mRNA. Since LTA is also a cell wall constituent of *S. gordonii* it may be implicated in the binding of *S. gordonii* to oral epithelial cells and stimulation of the production of IL-8 protein and hβD-2 mRNA.

Results show that *A. actinomycetemcomitans* SUNY 465 adheres to and invades oral epithelial cell line H357 to a greater degree than *A. actinomycetemcomitans* SUNY 462. The invasive strain also showed the ability to induce greater levels of hβD-2 mRNA expression and IL-8 secretion from oral epithelial cells. The number of invaded bacteria seen in oral epithelial cell line H357 for both strains is similar to that seen in the KB cell line (Meyer *et al.*, 1991) further demonstrating the ability of some *A. actinomycetemcomitans* strains to invade oral epithelial cells. The ability of *A. actinomycetemcomitans* SUNY 465 to adhere to oral epithelial cell line H357, as demonstrated in this chapter has been shown to be greater than that seen with KB cells (Meyer and Fives-Taylor 1994). The ability of *A. actinomycetemcomitans* SUNY 462 to adhere to oral epithelial cells has not been previously reported. The studies presented reveal that the less invasive strain *A. actinomycetemcomitans* SUNY 462 also adheres less to oral epithelial cells, suggesting that the ability to adhere to *A. actinomycetemcomitans* is closely related to invasive ability.

Gingival epithelial cells are the first line of cells encountering periodontal pathogens and as such it is important they rapidly respond to bacterial contact. The interaction between the oral periodontal pathogen *A. actinomycetemcomitans* and oral tissue may contribute to

the initial recruitment of neutrophils and immature dendritic cells due to IL-8 and hβD-2 production. It has previously been suggested that the invasive and binding properties of *A. actinomycetemcomitans* contribute to enhanced cytokine induction from primary oral epithelial cells and two oral cell lines (Huang *et al.*, 1998).

Whilst other studies have concentrated on *A. actinomycetemcomitans* binding and invasion of epithelial cells and IL-8 induction separately, the results of this study have shown the innate immune responses to invasive and non-invasive strains of *A. actinomycetemcomitans*. After 2h (the time at which adhesion and invasion were measured) incubation with both *A. actinomycetemcomitans* strains, only SUNY 465 induced IL-8 in oral epithelial cells. Increased exposure of 5h, as shown in Chapter 6, section 6.4.1.1 demonstrated that both strains induced IL-8, indicating that over a longer period direct association with oral epithelial cells is not necessary to stimulate an IL-8 response. In fact, the non-invasive *A. actinomycetemcomitans* strain SUNY 462 induced IL-8 to a greater extent at 5h exposure.

Other studies have shown, in agreement with these results that direct bacterial contact stimulates the release of cytokines, including the chemokine IL-8, from various epithelial cells. For example, HaCaT keratinocytes (skin derived) incubated with adherent *S. pyogenes*, but not non-adherent *S. pyogenes*, show increased expression of IL-1α, IL-1β and IL-8 (Wang *et al.*, 1997). Several studies have also demonstrated a correlation between bacterial adherence and/or invasion in gastric cells. A specific array of 4 proinflammatory cytokines, IL-8, MCP-1, TNF-α and GM-CSF, were co-ordinately expressed and upregulated in human colon epithelial cell lines in response to bacterial invasion or stimulation with TNF-α or IL-1 (Jung *et al.*, 1995). The regulated expression of a specific array of proinflammatory cytokines in colon epithelial cell lines, reported by this group, suggests that these cells are programmed to provide chemotactic and activating signals to

adjacent and underlying immune and inflammatory cells in the initial period following microbial invasion of epithelial cells. Further, production of proinflammatory cytokines by epithelial cells in response to TNF- α or IL-1 produced by other cells in the epithelial microenvironment would serve to amplify the mucosal inflammatory response. Whilst the present study has not investigated the cytokine profile of the H357 oral epithelial cell line, other studies have shown that TNF- α , IL-1, oral microbes and/or their products upregulate the production of IL-8, MCP-1, GM-CSF and TNF- α from oral epithelial cells (Bickel *et al.*, 1996, Li *et al.*, 1996, Dongari-Bagtzoglou and Kashleva, 2003, Han *et al.*, 2003, Uehara *et al.*, 2002). It could be suggested that since these cytokines, which have a well documented role in chemotaxis and activation of inflammatory cells, are expressed in response to bacterial invasion or other proinflammatory agonists, oral epithelial cells may be programmed to provide a set of signals for the activation of the mucosal inflammatory response in the earliest phases after microbial invasion.

To study the mechanism of IL-8 induction, established gastric carcinoma epithelial cell lines (AGS and Kato III) and well-defined *H. pylori* strains were used in a modified *in vitro* system (Rieder *et al.*, 1997). The experimental design enabled the investigators to prevent direct contact of bacteria to epithelial cells by use of a filter membrane, which did not block secreted bacterial products crossing the membrane. Data showed that the direct contact of the bacterial cell with the epithelial cell is necessary for optimal IL-8 production. Further results showed that not only live bacteria, but also metabolically inactive bacteria, increased IL-8 secretion, suggesting that the mechanism leading to IL-8 stimulation does not apparently rely on live bacteria and an active metabolism but needs an intact membrane conformation whereby the proteins on the bacterial surface are presented in a particular aggregation state. When *H. pylori* proteins are isolated, the complex or aggregation of these molecules is apparently changed, making them unable to stimulate gastric epithelial cells to increase IL-8 production. In addition, this supports the concept

that adherence is a multistep process mediated by different adhesins to different sites in the gastric tissue (Rieder *et al.*, 1997).

Further to these results there is also evidence of oral bacteria, whose direct contact with oral epithelial cells stimulates the release of cytokines. The periodontopathic bacterium *Eikenella corrodens* 1073 expresses a GalNAc-sensitive lectin-like adhesin (EcLS). In an adherence assay, EcLS was shown to play a role as the adhesin of this bacterium in adherence to KB cells, and stimulate the production of IL-6 and IL-8. Another study has demonstrated that the oral commensal *F. nucleatum* adheres to and invades human gingival epithelial cells and this is accompanied by high levels of IL-8 secretion from the epithelial cells (Han *et al.*, 2000).

Both *A. actinomycetemcomitans* SUNY 462 and *A. actinomycetemcomitans* SUNY 465 stimulated increased expression of h β D-2 mRNA in oral epithelial cells. However, a denser band, indicating increased expression of h β D-2 mRNA, was found after incubation with *A. actinomycetemcomitans* SUNY 465 at higher MOIs. This may be related to differences observed in adhesion and invasive properties of the two strains, in that, direct contact with epithelial cells may be required to activate innate immune responses. After increased exposure of 5h, as seen in Chapter 6, section 6.4.1.2, both *A. actinomycetemcomitans* SUNY strains stimulated the expression of h β D-2 mRNA in oral epithelial cells, particularly at higher MOIs. With this finding we see a similarity with IL-8. Whilst at 2h it seems direct contact with oral epithelial cells induces IL-8 secretion, as seen with *A. actinomycetemcomitans* SUNY 465, a longer incubation period of 5h shows IL-8 protein and h β D-2 mRNA induction by both *A. actinomycetemcomitans* strains.

The ability of both invasive and non-invasive bacteria to induce h β D-2 mRNA in two other epithelial models has recently been reported.

A model of skin infection has been developed to study the expression of h β D-2 mRNA. Multiple species of bacteria with various propensities to adhere to and invade keratinocytes, and those with various pathogenic potential for causing clinically superficial and invasive skin infections, were tested for their ability to induce h β D-2 mRNA (Dinulos *et al.*, 2003). However, no correlation was found between adherence to keratinocytes and the level of h β D-2 expression when cells were challenged with *S. epidermidis*, *S. aureus* and *E. coli* strains (Dinulos *et al.*, 2003).

It has also been shown that both enteroinvasive bacterial pathogens, *Salmonella enterica* serovar Dublin and enteroinvasive *E. coli* 029:NM and the non-invasive pathogen *Helicobacter pylori* induce the expression of h β D-2 mRNA in human intestinal epithelial cell lines and in human intestinal xenographs (O'Neil *et al.*, 1999). O'Neil *et al.*, suggest that upregulated production of h β D-2 during *H. pylori* infection *in vivo* may result from direct contact of *H. pylori* with the epithelium or, alternatively, from epithelial cell stimulation with IL-1 released during the course of the mucosal inflammatory response.

Further studies by the same group showed h β D-2 mRNA expression when AGS cells were stimulated with IL-1 α , TNF- α , IFN- γ or *E. coli* LPS or infected with enteroinvasive pathogens *S. enterica* serovar Dublin and *E. coli* 029:NM, non-invasive pathogen *H. pylori*, non-invasive *E. coli* DH5 α , or the Gram-positive non-invasive bacterium *S. bovis*. h β D-2 mRNA was markedly increased in those cells following stimulation with IL-1 α and to a lesser extent, after TNF- α stimulation. Infection with the highly invasive bacterium *S. enterica* serovar Dublin at an MOI equivalent to that of *H. pylori*, upregulated expression of h β D-2 mRNA in AGS cells to an extent similar to that of IL-1 α . Parallel

infections with invasive and non-invasive *E. coli* strains or with the Gram-positive non-invasive bacterium *S. bovis*, at the same MOI as *H. pylori*, resulted in either little or no change in hβD-2 mRNA expression (O'Neil *et al.*, 2000).

Taken together, these results show that both non-invasive and invasive bacteria whether pathogenic or not have different effects on hβD-2 mRNA levels in epithelial cells.

With regard to the results of the studies presented in this chapter, it is impossible to say if it is the direct contact of *A. actinomycetemcomitans* on oral epithelial cells that is responsible for the differences seen in hβD-2 mRNA expression and indeed the production of IL-8. Further studies on the bacteria may reveal differences in properties which may affect oral epithelial responses to infection with them.

Further studies, similar to those mentioned earlier, used by Rieder *et al.*, 1997 would demonstrate the importance of direct *A. actinomycetemcomitans* contact with oral epithelial cells on innate immune responses.

These findings show that, during the early stages of infection, direct contact with oral epithelial cells may be important in signalling innate immune responses to combat the invading microorganism. However, with increased exposure to periodontal pathogens other undetermined factors seem to prolong the release of IL-8 and hβD-2, suggesting direct contact with epithelial cells is not a prerequisite for a sustained immune response.

Chapter 8

Final discussion and conclusions

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New information on epithelial innate defence responses, the interaction of epithelia and the immune system and the interaction of epithelial cells with bacteria leads us to a view of the integrated role of the epithelium and the host response to infection.

Both constitutive and inducible antimicrobial peptides are expressed in the gingival epithelium, suggesting that it has a normal surveillance function as well as a specific role in innate immune response to infection. The role of natural antibiotics is only just beginning to be appreciated, with potential applications for enhanced natural expression or as new therapeutic agents. Their role may be especially important in the oral cavity where there is constant exposure to microbial challenges (reviewed in Dale *et al.*, 2001, Weinberg *et al.*, 1998). The expression of inflammatory cytokines in the periodontal diseases with relation to the human beta-defensins may also play an important part in innate epithelial immunity. The work presented in this thesis is based on the hypothesis that the loss of expression of the human beta-defensins may be a risk factor for the periodontal diseases. Results presented in this thesis have also demonstrated the expression of pro-inflammatory cytokines, IL-8 in particular, to determine if they may be linked to the expression of human beta-defensins.

8.1 Expression in gingival tissues

Results from these studies have demonstrated the expression of h β D-1 and h β D-2 protein in gingival tissues and that the expression levels are similar in healthy and diseased tissues. Both peptides were most abundant in the uppermost-nucleated layers of the epithelium. The pattern of expression of human β -defensins within the gingiva is consistent with its function as a microbial barrier in the stratified epithelial tissue. Other recent studies have also shown that β -defensins are expressed in healthy and diseased tissues and that the

pattern of expression observed is the same as the results found in this thesis (Dale *et al.*, 2001, Lu *et al.*, 2004).

The study presented in chapter 3 which evaluate the expression of mRNA for cytokines and the human beta-defensins in gingival health and disease showed there to be increased expression of h β D-1, h β D-2, IL-1 β , IL-6, IL-8 and IFN- γ mRNA in diseased tissues compared to controls, however, only the increases in IL-1 β , IL-6 and h β D-1 mRNA expression were found to be statistically significant. The studies presented here show that the human beta-defensins are present in both health and disease and underline the importance of the protective role of β -defensins against pathogenic microorganisms in oral epithelia.

Other studies performed subsequently to those carried out in this study have further shown the expression of mRNA for the human beta defensins in oral tissue. Dunsche *et al.*, 2001 and 2002 have demonstrated expression of h β D-1 mRNA and h β D-2 mRNA in all healthy gingival samples tested. However, the papers by Dunsche and colleagues demonstrate conflicting findings in clinically inflamed oral tissue. Expression of h β D-1 mRNA was found in all clinically inflamed oral tissue in the 2001 paper but was only found in 23 of 40 clinically inflamed oral tissues in their more recent paper. Likewise, mRNA for h β D-2 was detected in 17 of 18 inflamed oral tissues, compared to 22 from 40 inflamed oral tissue samples in the 2001 and 2002 papers, respectively. In their 2002 paper, the authors suggest that the reduced level of expression of these defensins in inflamed tissues might be merely a matter of infiltration by lymphocytes or other immunocompetent cells leading to a relative reduction in the percentage of epithelial cells expressing h β D-1 and h β D-2.

More recently, using a much greater number of clinical samples from the same oral site, it has been shown that little difference in expression of h β D-1 mRNA was seen between

healthy and diseased gingival tissues. Higher levels of h β D-3 mRNA expression correlated with healthy tissue status, and there was also a trend for increased h β D-2 in healthy tissue (Bissell *et al.* 2004). Also, recent studies have shown that the expression of h β D-1, -2 and -3 mRNAs are correlated with each other in the gingival tissues of young children (Saitoh *et al.*, 2004). In accordance with the results of this study, the expression levels of human beta-defensins have also been shown to vary greatly between individuals, both in diseased and healthy tissues (Bissell *et al.*, 2004, Saitoh *et al.*, 2004). Since the expression of human beta defensins is so variable between individuals it may be of more relevance to compare healthy and diseased tissue from the same person.

Similarly to the studies by Bissell *et al.*, and Saitoh *et al.*, 2004, the results of this study appears to show differential expression of β -defensin mRNA among the gingival tissue of patients, supported by the associations of expression among the two defensins. When one defensin is expressed at a high level, the other also tends to be expressed at a high level. This finding may reflect variation in the regulation of the expression of β -defensins from patient to patient, or genotypic diversity including differences in genomic copy number recently described for specific β -defensins, whereby levels of mRNA expression were correlated with the number of genomic copies of defensin genes (Bissell *et al.*, 2004, Hollox *et al.*, 2003).

While it appeared that the human beta-defensins may be upregulated in diseased compared to healthy tissue the results were not found to be statistically significant. Many factors may influence the expression of the human beta-defensins in both healthy and diseased gingival tissue. Whilst pro-inflammatory mediators are known to induce the expression of human beta-defensins, and are most often associated with diseased tissue, they are also present in healthy tissues, as are growth factors, which have also been shown to induce expression (Sorensen *et al.*, 2003, Bissell *et al.*, 2004). The expression profiles of human-beta defensins mRNA in healthy and diseased tissue may actually reflect down-regulation of

the defensins during disease. For example, down-regulation of h β D-1 and another antibacterial peptide, LL-37, was reported in patients with bacillary dysenteries and in *Shigella*-infected cell cultures (Islam *et al.*, 2001).

Genes encoding the components of the innate host defences may underlie susceptibility to recurrent infection due to genetic defects.

A recent observation by Putsep *et al.*, 2002, on patients suffering from morbus Kostmann, a severe congenital neutropenia, highlights the role of neutrophil antimicrobial peptides in periodontal disease. Patients with morbus Kostmann are treated by recombinant granulocyte-colony stimulating factor, which restores their levels of neutrophils. Despite this treatment, patients still have recurrent infections and periodontal disease.

Neutrophils and saliva from patients with morbus Kostmann were deficient in neutrophil antimicrobial peptide LL-37 and had reduced levels of the α -defensins HNP1-3. Epidemiological studies have also demonstrated that genetic factors play a significant role in EOP (Hart, 1994, Michalowicz, 1994). Genetic polymorphisms have now been identified in the genes encoding h β D-1 and h β D-2 and initial studies suggest an association of one polymorphism in h β D-1 with resistance to oral *Candida* infection (Jurevic *et al.*, 2002). This suggests a need for the development of rapid assays for such genetic polymorphisms, which will permit further analysis of expression of the defensin genes and their possible role in susceptibility to periodontal disease (Dale, 2002).

8.2 Regulation of human β -defensin 2 by bacterial products

Since studies of human beta-defensin protein and mRNA expression in healthy and diseased gingival tissue failed to support the proposed hypothesis the next step was to determine the regulation of these peptides in oral epithelial cells.

8.2.1 The use of OSCC H357 cell line as a model of the oral epithelium

The use of the H357 oral epithelial cell line seems to be a valuable and reproducible model for the study of human beta-defensin expression in the oral epithelium. Finding a reliable model for the study of innate response gene modulation, such as beta-defensins, in the oral cavity, is important. The process of extracting, isolating and culturing normal human oral epithelial cells (NHOECs) is difficult, time-consuming and, due to interpersonal variability, may lead to results with limited reproducibility.

Whilst Dale *et al.*, 2002 insist that normal oral gingival epithelial cells do not express h β D-2 mRNA in cell culture unless stimulated, Feucht *et al.*, 2003 have demonstrated otherwise. They have also shown that the immortalised cell line OKF6/Tert responds similarly to NHOEC when stimulated with *A. actinomycetemcomitans*. In agreement with the results of this study, OKF6/Tert cells demonstrated higher levels of basal h β D-2 mRNA expression compared to NHOECs.

Interestingly, the expression of h β D-2 mRNA has been found to be higher in oral squamous cell carcinomas compared to normal tissue (Sawaki *et al.*, 2002). Since the H357 cell line was derived from an oral squamous cell carcinoma, this could account for the expression of h β D-2 mRNA sometimes observed in unstimulated cells.

8.2.2 Regulation of human beta-defensins in host cells

The expression of h β D-2 mRNA has been demonstrated in the oral epithelial cell line H357, which was derived from a SCC of the tongue. Further, it was shown that these oral epithelial cells responded to *E. coli* LPS by increased production of IL-8 protein and h β D-2 mRNA. In addition to *E. coli* LPS, the cell stimulant PMA, *P. gingivalis* LPS, *S. sanguis* LTA, *E. coli* GroEL and *A. actinomycetemcomitans* SAM also upregulated the expression of h β D-2 mRNA in oral epithelial cells.

Prior to the results presented in this study there was no information on the presence of human beta-defensins in human blood cells. Other investigations have since found that human monocytes express mRNA for h β D-1 and h β D-2 and that they are regulated by *E. coli* LPS. h β D-1 and h β D-2 are antimicrobial peptides that had, at the time of the studies presented herein, been found exclusively in a variety of epithelial cells. Subsequent to the experiments carried out in this study the expression of h β D-1 and 2 mRNA by human monocytes, macrophages and dendritic cells have been reported (Duits *et al.*, 2002, Fang *et al.*, 2003).

The study by Duits *et al.*, 2002 found that monocytes, monocyte-derived-macrophages (MDM), and monocyte-derived-dendritic cells (DC) all express h β D-1 mRNA. h β D-1 mRNA expression by monocytes and MDM was increased after activation with IFN- γ and/or LPS in a dose- and time-dependent fashion. Monocytes, MDM, alveolar macrophages and DC showed a limited expression of h β D2 mRNA, which could only be increased in monocytes and alveolar macrophages by IFN- γ and/or LPS in a dose- and time-dependent fashion. Immunocytochemical staining demonstrated the expression of h β D-2 peptide by freshly isolated blood monocytes and alveolar macrophages in cytospin preparations (Duits *et al.*, 2002).

Fang *et al.*, 2003 have shown that in human peripheral blood cells there is no expression of h β D-1 and h β D-2 mRNA and that they are both inducible after incubation with *E. coli* LPS. However, the level of h β D-1 mRNA expression was much lower than found by Duits *et al.*, 2002; the expression of h β D-1 mRNA even with stimulation by LPS was much lower at 12h and was not detectable at 24h.

The results of chapter 4 demonstrate that whilst the upregulation of IL-8 protein and h β D-2 mRNA was blocked by pre-incubation with a CD14 antibody, flow cytometry failed to

detect CD14 on the cell surface. Further investigations demonstrated the presence of mRNA for TLR2 and TLR4, recent evidence from other research groups has highlighted the importance of these receptors in the induction of h β D-2. Results presented by Birchler *et al.*, 2001, show that HEK293 cells transfected with TLR2, but not wild type cells responded to stimulation with bacterial lipoprotein by production of h β D-2, more recent studies have shown similar results.

It has been shown that airway epithelial cells grown at an air-liquid interface responded to bacterial lipopeptide in a TLR2-dependent manner with induction of the expression of mRNA and protein for the antimicrobial peptide h β D-2 and IL-8 (Hertz *et al.*, 2003). TLR2 has also been shown to mediate the induction of h β D-2 through the NF- κ B pathway in response to bacterial LTA in human airway epithelia (Wang *et al.*, 2003). Wang *et al.*, 2003 suggested the working model that TLR can sense bacterial infection on the airway surface; activation of the TLR can lead to upregulation of antimicrobial peptides to facilitate elimination of the bacteria. At the same time, proinflammatory cytokine/chemokines can be induced and neutrophils and macrophages mobilized to the airway to help clear the infection (Wang *et al.*, 2003).

8.2.3 The validity of quantification of PCR products

The PCR programs used in this thesis were not optimised for the number of PCR cycles for each primer pair. Optimisation would have ensured that the reaction would have been stopped in the exponential phase. Only then would it have been valid to include quantification by densitometry, and ideally an internal standard should have been included. Also, if the band is to be compared to a housekeeping gene, cycle number for this would also need to be adjusted for the exponential phase. Relatively accurate methods have been developed since the work in this thesis was undertaken, this includes competitive PCR (MIMIC) or real-time PCR based on the use of fluorogenic probes. The MIMIC technique

requires an internal competitor that has to be specifically designed and then constructed for each specific RNA to be studied (Ali *et al.*, 1997, Marone *et al.*, 2001). An amplification curve with the internal competitor is then built for each sample. Quantification, while accurate, requires a relatively large amount of cDNA and a large number of amplification reactions per samples, moreover this technique requires intensive initial work when different RNAs have to be analysed and hence different mimic competitors need to be constructed (Marone *et al.*, 2001).

8.3 Induction of hβD-2 mRNA by oral bacteria

The results presented in this thesis also reveal that both the oral commensal *S. sanguis* and the periodontopathogens *P. gingivalis* and *A. actinomycetemcomitans* upregulate hβD-2 mRNA expression in oral epithelial cells. Recent evidence suggests that commensals chronically stimulate the innate immune system, keeping it on guard in certain places where injury would be followed by infection too rapidly for the adaptive immune system to respond (Krisanaprakornkit *et al.*, 2000). The results presented in this thesis further support this view.

Recently published studies demonstrate the effect of both oral commensal and potentially pathogenic bacteria on hβD-2 expression by oral keratinocytes, they also document the signalling pathways utilised by these bacteria.

Studies have shown that the JP2 *A. actinomycetemcomitans* strain induces hβD-3 gene expression but has no effect on hβD-2 mRNA in normal human oral epithelial cells nor an immortalised cell line derived from human oral epithelial cells (Feucht *et al.*, 2003). Furthermore, the work of this group indicates that an *A. actinomycetemcomitans* heat-labile factor may be responsible for the induction of hβD-3. It may be that, like hβD-2 as shown

in Chapter 5, h β D-3 is also upregulated by *A. actinomycetemcomitans* SAM in oral epithelial cells.

Other studies have also shown that *A. actinomycetemcomitans*, *S. gordonii* and *P. gingivalis* upregulate h β D-2 mRNA in cultured oral keratinocytes, and that the inhibitor of MAP kinase p38 reduces the level of h β D-2 mRNA (Chung and Dale, 2004). It was also shown that h β D-2 induction in oral keratinocytes by *P. gingivalis* and *A. actinomycetemcomitans*, was blocked by inhibitors of NF- κ B (Chung and Dale, 2004). These results indicate that commensals and pathogenic bacteria utilise different pathways in h β D-2 induction and suggest that epithelial cells from different body sites have common signalling mechanisms to distinguish between commensal and pathogenic bacteria (Chung and Dale, 2004). It could be that NF- κ B transcription factors may be involved in the h β D-2 mRNA upregulation seen in oral epithelial cell line H357 after contact with *A. actinomycetemcomitans* SUNY strains.

More recent studies by this research group have shown that viable intact *P. gingivalis* induced h β D-2 mRNA, but *P. gingivalis* cells washed in phosphate-buffered saline failed to induce h β D-2 in HOK at any MOI (Chung and Dale, 2004). The authors suggest that these findings imply a possible role of *P. gingivalis* proteases in h β D-2 mRNA induction.

It has also been shown that the invasive/adhesive ability of oral pathogens, in this case *A. actinomycetemcomitans*, may play a significant role in the organism's ability to upregulate inflammatory mediators. Studies presented in Chapter 7 show that the more invasive/adhesive strain of *A. actinomycetemcomitans* (SUNY 465) may upregulate h β D-2 mRNA and IL-8 protein in oral epithelial cells, compared to the less invasive/adhesive SUNY 462. Further studies would need to be conducted to determine whether this is as a consequence of invasion of oral epithelial cells by bacteria.

Other studies have shown that bacteria activate the transcription factors NF- κ B and MAP kinases upon invasion/adhesion to human cells. The interaction of *Neisseria meningitidis* with human brain microvascular endothelial cells, *S. aureus* with osteoblasts and *Listeria monocytogenes* with epithelial cells have all been shown to stimulate MAP kinase pathways (Tang *et al.*, 1994, Ellington *et al.*, 2001, Sokolova *et al.*, 2004). Whilst the interaction of *Neisseria gonorrhoeae* with epithelial cells leads to the activation of the transcription factor NF- κ B (Naumann *et al.*, 1997).

Since the upregulation of h β D-2 mRNA by *A. actinomycetemcomitans* in oral epithelial cells has been shown to involve the activation of NF- κ B (Chung and Dale, 2004), it could be that this is the case in the infection experiments demonstrated in these studies.

8.4 Therapeutic potential of antimicrobial peptides

Although much has yet to be learned regarding the exact role of defensins in protecting the gingiva from both the commensal and pathogenic members of the indigenous microbiota of the oral cavity, interest has already turned to the use of these peptides and their synthetic analogues in the prevention and treatment of disease.

Several reviews have suggested that β -sheet antibiotic peptides may be useful as dental therapeutics (Miyasaki, 1998, Weinberg, 1998 and Hancock, 1998). Porcine protegrins have been shown to have potent activity against oral bacteria such as *A. actinomycetemcomitans*, *Capnocytophaga* spp. and also anaerobic bacteria associated with adult periodontitis, including *P. gingivalis*, *P. intermedia* and *F. nucleatum* (Miyasaki, 1997, 1998). More recently there have been reports on the development of magainin analogues, which have also been found to have significant antimicrobial action against anaerobic oral pathogens (Genco *et al.*, 2003).

The viability of large-scale production of defensins and analogues that retain *in vitro* bactericidal effects against *C. albicans*, *P. gingivalis*, *A. actinomycetemcomitans*,

S. gordonii and *S. mutans* has already been shown (Raj *et al.*, 2000a, Raj *et al.*, 2000b). The same group has also gone on to show the possibility of linking a defensin analogue to an apatite binding protein which can then bind to hydroxyapatite, be slowly released, and still be effective against *C. albicans* and *A. actinomycetemcomitans* (Raj and Dentino, 2002).

It has recently been demonstrated that beta-defensins play an important role in the adaptive immune system. Reports have suggested that antimicrobial peptides and chemokines have overlapping functions (Durr and Peschel, 2002). Evidence, which demonstrates the overlapping functions of chemokines and defensins, shows that h β D-2 shares the CCR6 receptor for triggering chemotactic responses in dendritic cells and CD4/CD45RO (memory) T cells and CD8 T cells with the chemokine CCL20 (LARC) (Yang *et al.*, 1999). They may also play a role in allergy via increased histamine release and PGD₂ production by mast cells (Niyonsaba *et al.*, 2001).

Another study has highlighted the potential role of defensins in triggering the adaptive immune response. A mouse model was used to demonstrate the possibility of inducing an adaptive immune response using defensins co-administered intranasally with an antigen to prevent and/or treat disease (Brogden *et al.*, 2003).

8.5 Conclusions

The results of this study demonstrate that the human beta-defensins are expressed in gingival tissue at the superficial layer. This demonstrates that they are well placed to deal with pathogenic bacteria. Both h β D-1 and h β D-2 mRNA are expressed in healthy and diseased tissue probably due to the constant exposure of the tissue to both commensal and pathogenic bacteria. Further evidence supports this theory; results have shown that both commensal and pathogenic bacteria and their products are capable of upregulating the production of h β D-2 mRNA in oral epithelial cells. The expression of pro-inflammatory

cytokines may also be linked to the expression of human beta-defensins in diseased gingival tissue, and results have shown the co-expression of pro-inflammatory cytokines and the human beta-defensins compared to healthy gingival tissue. Results suggest that the invasive properties of oral bacteria may play a role in the further upregulation of h β D-2 mRNA in oral epithelial cells since the invasive strain of *A. actinomycetemcomitans* appeared to upregulate the expression of h β D-2 to a greater degree than the non-invasive strain (chapter 7, figure 7.17). However, results are not quantitative, and further investigations would be needed to determine if the invasive qualities of *A. actinomycetemcomitans* affects the expression of h β D-2 in oral epithelial cells. The upregulation of h β D-2 mRNA by a wide variety of components, bacterial or otherwise in oral epithelial cells may have therapeutic potential, however further studies would need to be carried out to determine the correlation between mRNA and protein expression of h β D-2.

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