Antimicrobial Peptides in the Oral Environment: Expression and Function in Health and Disease

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Abstract

The oral cavity is a unique environment in which antimicrobial peptides play a key role in maintaining health and may have future therapeutic applications. Present evidence suggests that α–defensins, β–defensins, LL-37, histatin, and other antimicrobial peptides and proteins have distinct but overlapping roles in maintaining oral health and preventing bacterial, fungal, and viral adherence and infection. The expression of the inducible hBD-2 in normal oral epithelium, in contrast to other epithelia, and the apparent differential signaling in response to commensal and pathogenic organisms, provides new insights into innate immunity in this body site. Commensal bacteria are excellent inducers of hBD-2 in oral epithelial cells, suggesting that the commensal bacterial community acts in a manner to benefit the overall innate immune readiness of oral epithelia. This may have major significance for understanding host defense in the complex oral environment.

The oral environment

The oral cavity is a unique environment. Oral mucosa is a critical protective interface between external and internal environments and must serve as a barrier to the myriad microbial species present in this warm, moist environment. The oral cavity is the only area of the body in which hard tissues break through the epithelial surface. The periodontal epithelium surrounding the tooth is specialized to form an attachment and seal around each tooth. This unique function imparts special challenges to the tissue and leads to certain vulnerabilities associated with periodontal disease, especially in view of the continual exposure to the bacterial biofilm (dental plaque) that forms on the tooth surface at the junction of the soft tissue. Thus, this anatomical region is one where there is a significant risk of bacterially induced infection and inflammation.

Antimicrobial peptides are important contributors to maintaining the balance between health and disease in this complex environment. These include several salivary antimicrobial peptides, the β–defensins expressed in the epithelium, the α–defensins expressed in neutrophils, and the cathelicidin, LL-37, expressed in both epithelium and neutrophils. These peptides are part of the host innate immune response in this environment. Epithelia, polymorphonuclear leukocytes (neutrophils), and saliva all contribute to the maintaining the health of the oral cavity in overlapping but independent ways. This review will focus on the human oral cavity and include 1) the expression and function of antimicrobial peptides in the oral cavity in the context of innate immune responses, 2) regulation of β–defensins which has led to advances in our understanding of oral epithelial innate immunity, and 3) functional efficacy against oral microbes when it is known.

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Epithelial antimicrobial peptides

Historically, the oral epithelium has been considered mainly as a passive covering that becomes damaged and ulcerated in disease. This view has changed dramatically and the epithelial compartment is now seen as providing both a physical barrier to infection and playing an active role in innate host defense (Dale, 2002; Darveau *et al*., 1997; Ganz, 2003; Tonetti, 1997). Epithelial cells are in constant contact with bacterial products from supra- and sub-gingival biofilms on the tooth surface as well as from bacteria attached to mucosal surfaces. These cells respond to bacteria in an interactive manner; they secrete IL-8 and other chemokines and cytokines to alert various cell types and attract neutrophils. They also produce natural antimicrobial peptides and proteins constitutively and inducibly in response to bacterial exposure. These antimicrobial peptides are part of the innate immune system, a complex set of responses that keeps microbial invaders in check and maintains the microbial ecology of the healthy mucosa (Weinberg *et al*., 1998). Thus, the epithelium functions to actively respond to the environment, participates in response to infection, in signaling further host responses, and in integrating innate and acquired immune responses.

The first antimicrobial peptide identified in oral epithelium was the β-defensin, lingual antimicrobial peptide (LAP), described in bovine tongue (Zasloff *et al*., 1995). We now know that several families of natural antibiotic peptides or proteins are expressed in oral epithelium. These include members of the β-defensin family of peptides, the protein calprotectin (also known as calgranulin), and the multifunctional peptide, adrenomedullin (Dale and Krisanaprakornkit, 2001; Devine, 2003; Ganz, 2003; Kapas *et al*., 2001a; Ross and Herzberg, 2001). These antimicrobials have broad specificity with activity against Gram-positive and Gram-negative bacteria (reviewed in Hancock, 1997), as well as against yeast and some viruses (Ganz, 2003; Lehrer and Ganz, 2002; Quinones-Mateu *et al*., 2003). They complement the antimicrobial factors of saliva, such as the histatins, lysozyme, and salivary immunoglobulins. Both constitutive and inducible antimicrobial peptides are expressed in gingival epithelium, suggesting that they

have a normal surveillance function as well as a specific role in innate host defense in response to infection. The role of these 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 for the oral cavity in which there is constant exposure to microbial challenges (Dale and Krisanaprakornkit, 2001; Weinberg *et al*., 1998). Further, individual variation in expression may contribute to disease susceptibility. Overall expression of antimicrobial peptides in the oral cavity is summarized in Table 1.

β-*Defensins in oral cells and tissue*

Human β-defensins (hBDs) are expressed in all human epithelial tissues tested to date including those of the oral cavity (Dale and Krisanaprakornkit, 2001; Lehrer and Ganz, 2002). β–defensins are expressed in gingiva, tongue, salivary glands, and mucosa (Mathews *et al*., 1999). They are present in oral inflammatory conditions, oral carcinomas, and some cell lines derived from oral carcinomas (Abiko *et al*., 1999; Mizukawa *et al*., 2000). Oral keratinocytes have been a useful model for investigation of the regulation of β–defensin expression. The human peptide hBD-1 is constitutively expressed while hBD-2 and hBD-3 are upregulated in inflamed skin and other epithelia (Diamond *et al*., 1991; Harder *et al*., 1997a; Harder *et al*., 2001; Krisanaprakornkit *et al*., 2000; Krisanaprakornkit *et al*., 1998; Liu *et al*., 2002; Schonwetter *et al*., 1995; Singh *et al*., 1998; Stolzenberg *et al*., 1997; Valore *et al*., 1998; Zhao *et al*., 1996). These and similar peptides in other mammalian species are induced by inflammation and proinflammatory cytokines, and in some cell types, by bacterial lipopolysaccharide (LPS) (Diamond and Bevins, 1994; Diamond *et al*., 2000; Diamond *et al*., 1996; Russell *et al*., 1996). Oral epithelial tissues and oral epithelial cells derived from gingiva express hBD-1, -2, and -3 (Dunsche *et al*., 2002; Dunsche *et al*., 2001; Krisanaprakornkit *et al*., 2000; Krisanaprakornkit *et al*., 1998). Oral expression of other members of the large β-defensin family recently identified by Schutte and coworkers is not yet characterized (Schutte *et al*., 2002).

β-*Defensins in oral tissue.* Normal uninflamed gingival tissues express both hBD-1 and hBD-2. Both the constitutive and inducible β-defensins have been found in essentially all gingival biopsies tested, both healthy uninflamed and inflamed samples (Krisanaprakornkit *et al*., 2000; Krisanaprakornkit *et al*., 1998). Thus, normal non-inflamed oral epithelial tissue is activated to express hBD-2, in contrast to normal epidermis, trachea, and gut. This expression of hBD-2 is not accompanied by upregulation of markers of host innate immune response such as IL8. It seems to be part of the normal barrier function of gingival epithelia. The hypothesis is that this exposure represents a useful interaction between the commensal bacteria and the tissue, resulting in enhanced expression of hBD-2 and therefore providing an advantage in subsequent response to other potentially pathogenic organisms. This may be a general paradigm, since it has been shown that *Staphylococcus epidermidis*, the major commensal skin bacterium, also is an excellent inducer of hBD-2 in epidermal keratinocytes (Chung and Dale, 2004; Dinulos *et al*., 2003). The role of hBD-1 may be in preventing commensal bacteria from becoming opportunistic pathogens, while hBD-2 and hBD-3 and other inducible antimicrobials may be more effective against pathogens.

β-*Defensins are differentially regulated in oral keratinocytes.* HBD-1 is constitutively expressed in oral keratinocytes while hBD-2 is upregulated by bacterial and proinflammatory stimuli (Krisanaprakornkit *et al*., 2000; Mathews *et al*., 1999). The unstimulated level of hBD-2 is extremely low and upregulation of mRNA expression occurs rapidly, within 2–4 hr after stimulation with bacterial products or TNFα. Stimulants include TNFα, IL1β, *Fusobacterium nucleatum* (a commensal oral bacteria, not associated with periodontal disease), and *Porphyromonas gingivalis,* a periodontal pathogen. Nevertheless, bacterial LPS preparations from *F. nucleatum, P. gingivalis* and *E.coli* are relatively poor inducers of hBD-2 in oral keratincytes (Krisanaprakornkit *et al*., 2000). This is in contrast to regulation in tracheal epithelial cells in which hBD-2 is upregulated by *E. coli* LPS (Becker *et al*., 2000; Diamond *et al*., 2000). HBD-3 is

Table 1. Antimicrobial peptides expressed in the oral cavity

Antimicrobial peptide	Site of expression	Role/comments[1]
HNP1–4 (α-Defensins)	Neutrophils (azurophilic granules) Gingival sulcus Sites of inflammation	Antibacterial, antifungal, and antiviral. Functional levels in GCF Expression defective in Morbus Kostmann syndrome (congenital neutropenia associated with periodontal disease)
LL-37	Neutrophils Gingival sulcus Saliva	Primarily antibacterial. Expression is defective in Morbus Kostmann syndrome.
β-Defensins hBD-1 hBD-2 hBD-3	Suprabasal layers of stratified epithelium; hBD-1 and hBD-2 absent in junctional epithelium; hBD-3 mRNA is widely expressed but peptide localization is not known	Antibacterial, antifungal, and antiviral. Part of the protective barrier function of epithelium. Secreted, may be associated with cell or mucosal surface. Also found in salivary glands and saliva.
Histatin	Saliva (parotid and submandibular)	Antifungal. Histidine-rich group of peptides. Histatin 5 (24 amino acids) is most active. Antifungal action requires metabolic activity.
Adrenomedullin	Epithelium	Antibacterial, mitogenic, vasodilator, inducible peptide.

[1] See text for references.

upregulated by interferon-γ (Garcia *et al*., 2001), various bacteria (Harder *et al*., 2001), and by *Actinobacillus actinomycetemcomitans* in oral epithelial cells (Feucht *et al*., 2003).

β-Defensin expression is associated with differentiation. HBD-1 and hBD-2 mRNA and peptides are expressed as a function of differentiation in cultured oral keratinocytes. In keratinocytes *in vitro*, the peptides are detected only in cells that are expressing involucrin, an early marker of differentiation (Dale *et al*., 2001; Dale and Krisanaprakornkit, 2001). In normal gingival tissue mRNAs for both hBD-1 and hBD-2 are most strongly expressed in the spinous layer of the tissue, while the peptides are detected in the upper spinous, granular, and cornified layers. The strongest expression is at the gingival margin, adjacent to the region of plaque formation on the tooth surface and in inflamed sulcular epithelium (Dale *et al*., 2001). The tissue location is consistent with a role for these peptides in the epithelial antimicrobial barrier. HBD-1 and hBD-2 are not detected in junctional epithelium although this region is frequently the site of inflammation. However, the cells of the junctional epithelium are relatively undifferentiated in contrast to other regions of the oral cavity (Schroeder and Listgarten, 1997). The lack of expression in the junctional epithelium, the suprabasal localization in stratified epithelia, and the association with differentiation *in vitro*, all point to a dependence on normal differentiation for expression of β–defensins in stratified oral epithelia as well as in the epidermis (Liu *et al*., 2002).

Epithelial antimicrobials

Calprotectin. Calprotectin or calgranulin is a heterodimeric calcium- and zinc-binding protein, also referred to as S100A8 and S100A9. It is expressed in neutrophils, monocytes, macrophages, and mucosal keratinocytes and is involved in leukocyte trafficking and arachidonic acid metabolism (Nacken *et al*., 2003). It is upregulated in inflammatory conditions, including periodontal disease in which it is elevated in the gingival cervicular fluid (Kido *et al*., 1999). It is constitutively expressed in cells of stratified oral epithelia and in cultured gingival epithelial cells (Ross and Herzberg, 2001) and is highly responsive to stress in epidermis (Marionnet *et al*., 2003). The mechanism of antimicrobial action of calprotectin appears to be due to competition for zinc, a growth requirement for many microbial species (Brandtzaeg *et al*., 1995). Calprotectin expression confers protection from bacterial binding and invasion and may contribute to resistance of gingival cells to invasion by *Porphyromonas gingivalis*, a gram-negative periodontal pathogen (Nisapakultorn *et al*., 2001).

Adrenomedullin. Adrenomedullin is a multifunctional peptide that was initially characterized for its vasodilatory effects and subsequently has been recognized to have antibacterial function against both Gram positive and Gram negative bacteria from the oral cavity, skin, respiratory tract and gut (Allaker and Kapas, 2003). It does not display antifungal activity. It is constitutively expressed and secreted by oral epithelial cells; expression is also increased in response to live oral bacteria, IL1, and TNFα (Kapas *et al*., 2001a; Kapas *et al*., 2001b). Adrenomedullin consists of 52 amino acids with one intramolecular disulfide bond. The precursor is encoded by the ADM gene which maps to human chromosome 11. Although there are some functional parallels to the β–defensins, the adrenomedullin gene and protein structure differ from that of the β–defensins. Adrenomedullin has some homology with calcitonin gene-related peptide (Kitamura *et al*., 1993) and binds to the calcitonin receptor-like receptor (McLatchie *et al*., 1998) and is thought to have hormone-like functions in the control of circulation. Mice lacking expression of adrenomedullin die in mid-gestation due to cardiovascular abnormalities (Caron and Smithies, 2001).

Epithelial and neutrophil antimicrobial peptides function together in gingiva

Neutrophil antimicrobial peptides work together with the epithelium to provide a barrier to microbial colonization in the oral cavity, particularly in the region adjacent to the tooth surface. The junctional epithelium which forms the attachment of the soft tissue to the tooth surface is less differentiated and more permeable than the sulcular or oral surface epithelium. Neutrophils migrate through the junctional epithelium in response to a gradient of IL8 expressed in the junctional and sulcular epithelia (Tonetti *et al*., 1998). The importance of neutrophils in oral health is readily seen by the severity of problems in individuals with defects in neutrophil chemotaxis or function. Such defects are associated with severe periodontal disease occurring at a young age (see below).

LL-37 and α-defensins

The cathelicidin, LL-37, is expressed in epithelial cells especially following inflammatory stimulation, and both the protein and mRNA have been detected in human tongue and buccal mucosa (Frohm Nilsson *et al*., 1999) and saliva (Murakami *et al*., 2002). However, LL-37 detected in gingival epithelium by immunohistochemisty appeared to be the product of neutrophil migration through the tissue rather than the epithelial cells per se (Dale *et al*., 2001). In addition, the neutrophil α–defensins, HNP-1–3, are readily detected in the junctional epithelium (Dale *et al*., 2001). They can be detected in quantities consistent with their antimicrobial function in the gingival crevicular fluid that lies between the epithelium and the tooth surface (McKay *et al*., 1999).

Localized expression of antimicrobial peptides in gingiva

The α- and β-defensins and LL-37 are localized in different sites in the gingiva, suggesting that they may serve different roles in the several ecological niches of the periodontium. The β-defensin peptides, hBD-1 and hBD-2 are localized in the differentiated layers of the gingival epithelium in a pattern of expression consistent with function as a microbial barrier (Dale *et al*., 2001). Their expression is particularly strong at the gingival margin where the tissue can be expected to be in nearly continuous contact with supragingival plaque. While β-defensins are poorly expressed in the undifferentiated cells of junctional epithelium, the α-defensins and LL-37

are present in high amounts in neutrophils that migrate through the junctional epithelium to the gingival sulcus. Thus, junctional epithelium is protected by α-defensins and LL-37 released from neutrophils, whereas the differentiated stratified epithelia are protected by β-defensins (Dale *et al*., 2001; McKay *et al*., 1999). Taken together in the context of the oral cavity, the observations on tissue and gingival fluid suggest that the gingiva is protected by α– and β–defensins, LL-37, and salivary antimicrobial peptides as summarized in Fig. 1. This is quite analogous to the findings in intestine in which the crypts are protected by α–defensins and the epithelia of intestinal villae express β-defensins (Bevins *et al*., 1999). Both α- and β-defensins, as well as LL-37, signal other innate and acquired immune responses (see below, reviewed in Lehrer and Ganz, 2002) in addition to their antimicrobial properties.

The expression and regulation of antimicrobial peptides indicates heightened innate immune responses in oral epithelia

HBD-2 is expressed in both normal and inflamed oral epithelia

In contrast to most other epithelia, in which hBD-2 is expressed only in the presence of infection or inflammation (including skin, trachea, gut epithelium), this peptide is expressed in normal *uninflamed* gingival tissue (Dale *et al*., 2001). Present evidence suggests that the high level of hBD-2 expression is due to the exposure of the tissue to commensal, non-pathogenic bacteria (Krisanaprakornkit *et al*., 2000) and *that the innate immune responses of normal oral epithelia are at a heightened state of readiness*. This has been most clearly demonstrated in studies of β-defensin expression in oral epithelial cells *in vitro*. Normal oral gingival epithelial cells do not express hBD-2 in cell culture unless stimulated. Commensal and pathogenic bacteria, proinflammatory cytokines and phorbol ester, a general cell activator, are all effective stimulants. Cell wall preparations of the commensal organism, *F. nucleatum,* are particularly effective in up-regulating hBD-2 mRNA expression (Krisanaprakornkit *et al*., 2000).

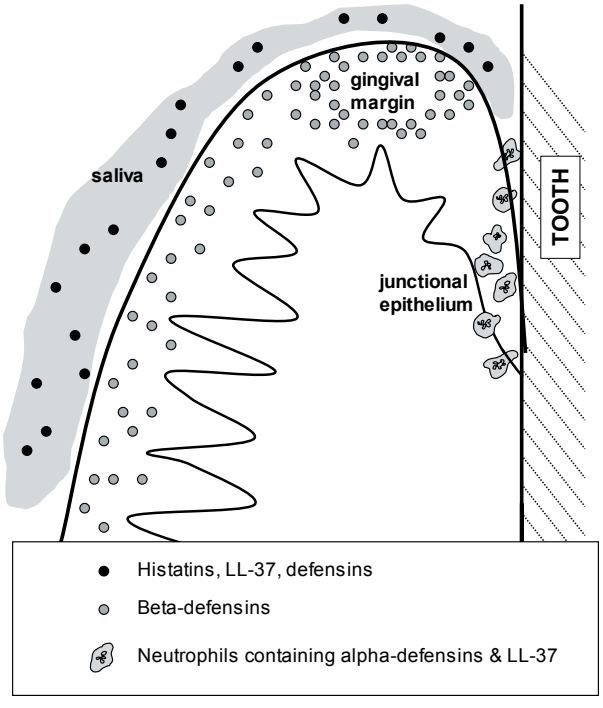

Fig. 1 Localized expression of antimicrobial peptides in gingiva. Diagrammatic view of a cross-section of the gingiva illustrating the oral aspect of the tissue, the gingival margin at the crest of the gum tissue, and the junctional epithelium attaching to the tooth surface. The sulcular epithelium is adjacent to the space (sulcus) between the tissue and the tooth. Neutrophils, containing α–defensins and LL-37, migrate through the junctional epithelium into the gingival sulcus. β–Defensins are expressed in the gingival epithelium. The tissue is bathed in saliva containing histatins, LL-37 and α– and β–defensins.

Signaling pathways for hBD-2 in oral epithelial cells

HBD-2 up-regulation in response to oral commensal bacteria does *not* seem to utilize the NF-κB intracellular signaling cascade typically associated with recognition of bacterial products, but instead utilizes the JNK and p38 mitogen activated protein kinase (MAPK) pathways that are associated with cytokine and stress responses (Krisanaprakornkit *et al*., 2002). Intracellular calcium signaling is also involved (Krisanaprakornkit *et al*., 2003). Utilization of this signaling pattern has now been extended to show that commensal organisms from both oral and skin sites do not utilize the NF-κB pathway for hBD-2 up-regulation, in contrast to pathogens which use an NF-κB pathway (Chung and Dale, 2004). This differs from results in other tissues, for example, tracheal epithelium in which NF-κB signaling has been demonstrated (Diamond *et al*., 2000). This signaling pathway may represent an adaptation to the normal population of both Gram positive and Gram negative bacteria within the oral cavity. Similarly, colon epithelial cells upregulate hBD-2 expression poorly in response to LPS (O'Neil *et al*., 1999). The findings in oral epithelial cells are consistent with the hypothesis that oral epithelia are capable of distinguishing commensal and pathogenic organisms, that immune response signaling mechanisms in the presence of commensal bacteria differ from those in response to pathogens, and that the expression of hBD-2 is part of this scenario. Because commensal bacteria are excellent inducers of hBD-2 in oral epithelial cells, these findings suggest that the oral commensal bacterial community acts in a manner to benefit the overall innate immune readiness of oral epithelia. This may have major significance for understanding host defense in the complex oral environment. The receptors, molecular mechanisms and signaling pathways responsible for the distinction are not yet fully understood. Further, interpretation of various experimental approaches may be complicated by cross-talk between various receptor-mediated signaling pathways.

Epithelial receptors for hBD-2 regulation

Mammalian cells sense the presence of bacteria via Pattern Recognition Receptors, such as the Toll-like receptor or TLR family (Medzhitov, 2001). Lipoteichoic acid and peptidoglycans of Gram positive organisms and LPS of Gram negative bacteria signal via TLR2 and TLR4, respectively, activating NF-κB transcription factors and the JNK pathways that mediate up-regulation of multiple

innate and inflammatory responses. TLR2 and -4 are expressed on oral epithelial cells and up-regulated with interferon gamma stimulation (Uehara *et al*., 2002b). The LPS binding factor, CD14, a cofactor in the association of bacterial LPS to TLR4, is poorly expressed on epithelial cells but is present in soluble form in plasma. HBD-2 regulation in tracheal epithelial cells occurs via CD14, and NF-κB-mediated signaling (Becker *et al*., 2000; Diamond *et al*., 2000), and/or via TLR2-mediated signaling (Hertz *et al*., 2003). However, there is not yet convincing evidence that this pathway is involved in hBD-2 regulation in oral epithelial cells.

A second family of recently described receptors is the proteinase-activated receptors or PARs (Coughlin, 2000; Mackie *et al*., 2002). PARs act as sensors of possible danger, have a role in inflammation, and may also have a central role in the recognition of, and response to, bacteria within the oral cavity (Mackie *et al*., 2002), including regulation of the inducible β–defensins. Because the three major Gram-negative pathogens associated with periodontal disease, *P. gingivalis*, *Bacteroides forsythus* (recently renamed *Tannerella forsythensis*) and *Treponema denticola* (Socransky *et al*., 1998) each have proteases as part of their virulence mechanisms (Curtis *et al*., 2001; Fenno *et al*., 2001; Saito *et al*., 1997); these receptors may aid in differentially stimulating epithelial cell responses to pathogenic vs. commensal oral bacteria. PARs are implicated in platelet activation and response to injury via thrombin and trypsin (Lourbakos *et al*., 2001; Uehara *et al*., 2002a). PARs are seven-transmembrane domain G-protein coupled receptors. PAR activation involves proteolytic cleavage of the extracellular domain, resulting in a new amino-terminus that acts as a tethered ligand that binds to one of the extracellular loops of the receptor (Coughlin and Camerer, 2003; Mackie *et al*., 2002). PAR-1, -3, and -4 are activated by thrombin; PAR-2 is activated by various trypsin-like enzymes, including mast cell tryptase, neutrophil proteinase 3, as well as *P. gingivalis* proteinases (Lourbakos *et al*., 1998). These receptors can also be activated by peptide agonists that mimic the proteolytically produced amino-terminus. A PAR-2 peptide agonist up-regulated hBD-2 mRNA in oral epithelial cells and *P. gingivalis* mutants lacking proteinase fail to upregulate hBD-2, suggesting a role for PAR-2 in regulation of hBD-2 expression (Chung *et al*., 2004).

Other functions of antimicrobial peptides in the oral cavity

There is a growing recognition that the functions of chemokines and antimicrobial peptides overlap (Cole *et al*., 2001; Durr and Peschel, 2002). Epithelial cells signal Langerhans cells, the antigen presenting cells within epithelia, via cytokines, chemokines, and β-defensins, to connect innate immune responses with acquired immunity (Banchereau and Steinman, 1998; Yang *et al*., 1999). HBD-1 and hBD-2 act as chemoattractants for dendritic cells and T-cells. They also act as ligands for the receptor CCR6, a G-protein coupled receptor located in immature dendritic cells. Activation of the receptor results in dendritic cell maturation (Yang *et al*., 1999). Interestingly, the natural ligand for CCR6 is the chemokine CCL20 which has been shown to have structural similarity to hBD-2 (Hoover *et al*., 2002) and has antimicrobial activity (Starner *et al*., 2003). HBD-2 has also been reported as a ligand for TLR4 in dendritic cells (Biragyn *et al*., 2002), however, this finding is somewhat controversial and could be due to the ability of cationic hBD-2 to efficiently bind and act as a carrier for LPS delivery to the TLR4 receptor. HBD-2 also has effects on mast cells resulting in histamine release and intracellular Ca^{++} mobilization in a G protein-phopholipase C dependent manner (Niyonsaba *et al*., 2001).

The antimicrobial peptide LL-37 is a chemoattractant for neutrophil, monocytes and T-cells (Chertov *et al*., 1996; Yang *et al*., 2000). It also stimulates mast cells and alters macrophage gene expression to upregulate chemokines and their receptors resulting in greater responsiveness to the environment (Scott *et al*., 2002; Scott *et al*., 2000). HBD-2 also up-regulates gene expression for numerous cytokine and chemokine receptors in oral epithelial cells in a manner reminiscent of LL-37 (Yin and Dale, manuscript in preparation). Thus, hBD-2 enhances epithelial cell responses and alters gene expression in oral epithelial cells as well as mast cells and dendritic cells.

α–Defensins also have multiple functions as was recently reviewed (Yang *et al*., 2002). They selectively attract naïve CD4+ T-cells and immature dendritic cells via a G-protein coupled receptor (Yang *et al*., 2000). α–Defensins also stimulate mast cell degranulation (Befus *et al*., 1999), regulate complement activation (Prohaszka *et al*., 1997; van den Berg *et al*., 1998), and enhance macrophage phagocytosis (Ichinose *et al*., 1996).

Together these observations support the view that antimicrobial peptides promote adaptive immunity against microorganisms by recruiting both naïve T-cells, memory T-cells and antigen-presenting dendritic cells to regions of infection and stimulate repair and clean-up of infected sites via effects on mast cells and macrophages. In the oral cavity, this is an important ongoing process, particularly in the region of the gingival sulcus.

Salivary antimicrobial peptides

Antimicrobial components of the saliva (excluding salivary IgA) are generally referred to as the non-immune factors, but can be considered as part of the innate immune system of gene encoded factors that aid in protection against oral microbial colonization and infection (reviewed by Nieuw Amerongen and Veerman, 2002). These include a number of proteins such as salivary peroxidase, lysozyme, lactoferrin, cystatins, SLPI, agglutinin and mucins as well as peptides of the histatin family. In addition, the cathelicidin, LL-37, and α- and β–defensins are expressed and secreted by salivary glands and/or ducts.

α- and β-Defensins

β-Defensins have been demonstrated in salivary duct cells, but not acinar cells by immunohistochemical means (Sahasrabudhe *et al*., 2000) and in salivary glands and saliva by protein and RT-PCR analyses (Bonass *et al*., 1999; Mathews *et al*., 1999; Sahasrabudhe *et al*., 2000). HBD-1 mRNA has been identified in all major and minor salivary gland samples, while hBD-2 mRNA has been identified in a portion of samples, possibly associated with inflammation (Bonass *et al*., 1999). The secretion of β–defensins in saliva as well as their secretion from

epithelial cells (Diamond *et al*., 2001) may contribute to the protection of oral mucosal surfaces. Levels of β–defensins in saliva have not been quantified. The α–defensins, HNP1–3, have also been detected in saliva and are elevated in patients with oral inflammation (Mizukawa *et al*., 1999). Levels of HNP1–3 vary in healthy individuals ranging from undetectable to ~12 μg/ml (Goebel *et al*., 2000; Mizukawa *et al*., 1999). The presence of α–defensins in saliva is most likely derived from neutrophils and is a reflection of gingival or mucosal inflammation and loose or exfoliating teeth.

LL-37

Expression of the mouse cathelicidin CRAMP was detected in salivary acinar cells of submandibular and palatine minor glands (mRNA and protein), ducts cells (protein) and mucosa in both embryonic and adult mice. In the human, LL37 was detected in saliva (Murakami *et al*., 2002). The results suggest localized expression of different antimicrobial peptides in salivary glands and ducts, similar to that of the gingiva (see above).

Histatins

Histatins are a family of at least 12 histidine-rich, cationic peptides synthesized by parotid and submandibular salivary duct cells and present in saliva (reviewed by Devine, 2002). Multiple histatins are generated from proteolytic cleavage of histatin 1 (38 amino acids) and 3 (32 amino acids). Histatins 1, 3, and 5 comprise approx. 85% of the total histatin protein in saliva. Histatins exhibit antifungal properties *in vitro* with histatin 5 having the most potent fungicidal activity (Oppenheim *et al*., 1988). The HTN genes map to chromosome 4q13, and the histatins are expressed only in salivary glands (vanderSpek *et al*., 1989), in contrast to the more general expression pattern of other antimicrobial peptides.

Histatins and oral candida. Saliva plays a critical role in regulating *Candida* adhesion to mucosal surfaces (Ueta *et al*., 2000). A high frequency of oral candidiasis occurs in patients with reduced salivary flow, such as in patients with Sjögren's syndrome and histatins appear to be a main player in control of oral *Candida* (Jainkittivong *et al*., 1998). The fungicidal action of histatins requires active mitochondria (Helmerhorst *et al*., 1999a). α-defensins and histatins share some aspects of their antifungal action which is associated with ATP transport and reaction with purinergic-like cell surface receptors (Edgerton *et al*., 2000). The relationship of histatins and α- and β-defensins with oral *Candida* is not clear, but these peptides may work synergistically.

Altered antimicrobial peptide expression in the oral cavity

The innate immune system is essential in preventing infection, and the relationship of antimicrobial peptides and an individual's susceptibility to infection is an important area of investigation. A current approach to identification of protein function is to identify the physiologic consequences in null mice lacking expression of the gene under investigation. This approach has led to advances in the understanding of the role of LL-37 in systemic and skin infection by *S. aureus* (Nizet *et al*., 2001). However, because there are numerous defensins, it is not possible to unequivocally prove their importance by knock out technology in a mouse model system. An alternate approach was used by Wilson and coworkers (Wilson *et al*., 1999) in which they disrupted the gene for matrilysin, the enzyme required for activation of Paneth cell α–defensins in the intestine. A second approach is to take advantage of experiments of nature – via genetics and the occurrence of genetic disorders.

Neutrophil defects and defects in expression of α-defensins and LL-37

It has been known for many years that periodontal disease in prepubertal children is associated with defects in neutrophil function (Page *et al*., 1985). With a general neutrophil defect, for example in the chemotactic response, or in cyclic neutropenia, periodontal disease may occur in association with increased occurrence of otitis media and other repeated infections (reviewed by Hart and Kornman, 2000). Recently a genetic form of periodontal disease (PD) in young people, Morbus Kostmann syndrome, was shown to have a deficiency in the α–defensins, HNP1–3, and a near absence of LL-37 (Putsep *et al*., 2002). This led to the suggestion that LL-37 may be particularly important in its effects vs. the Gram-negative, *Actinobacillus actinomycetemcomitans*, an organism associated with rapidly progressive PD especially in young people.

Another early onset inherited form of PD is the Papillon-Lefevre syndrome in which the genetic defect is in the gene for cathepsin C, a lysosomal protease (Hart *et al*., 1999; Toomes *et al*., 1999). Periodontal disease and palmar/plantar hyperkeratosis occur in this disorder but without an associated high frequency of other types of infections. Apparently a specific effect of cathepsin C is important in each of these body sites, however, the key substrate(s) for cathepsin C in neutrophils and epidermis is(are) not known. To date, altered processing of antimicrobial peptides has not been examined in this disorder, but is among the possible candidate substrates.

Genetics and the defensins

HBD-1 polymorphism and oral candida carriage. Occurrence of genetic polymorphisms offers another means to ask questions about the role of antimicrobial peptides in oral health and disease. Multiple single nucleotide polymorphisms (SNPs) have been identified in the DEFB1 and DEFB2 (now renamed DEFB4) genes, encoding hBD-1 and hBD-2, respectively (Dork and Stuhrmann, 1998; Jurevic *et al*., 2002; Vatta *et al*., 2000). Such SNPs may alter the expression or function of defensins and could lead to altered susceptibility to infection. The SNPs, especially those that had a reasonably high frequency, occur mainly in promoter and untranslated regions of these genes. These SNPs could potentially alter the amount of the peptide expressed, but not the peptide itself. Individuals with Type 1 diabetes are susceptible to oral *Candida* infection and have marginal immunosuppression, therefore, these minor differences in β-defensin genes may have significant effects in this

population. The diabetic population had nearly 3-fold higher average oral *Candida* carriage than a non-diabetic group. However, in both populations, a SNP in DEFB1 (-44 C to G) was found to be associated with *protection* from oral *Candida* carriage. In other words, people who had the SNP had very low levels of oral *Candida.* The difference in relative risk for high *Candida* carriage was 25 ($p<0.01$) for the diabetic population and 8.5 for the non-diabetic group (Jurevic *et al*., 2003). The most obvious interpretation of this finding is that this SNP alters hBD-1 expression. Initial results using a molecular approach to address this possibility strongly suggest that the SNP results in increased hBD-1 protein expression, and this may explain the basis of the apparent protection from oral *Candida* carriage. Alternatively, the SNP may be linked to a genetic change in another functional gene in this chromosomal region. Nevertheless, the results suggest that individuals who are immunosuppressed, and thus more susceptible to opportunistic infections, may have greater reliance on innate immune defenses than healthy individuals who have multiple ways to fight infection.

Defensin gene duplications. Hollox and co-workers (Hollox *et al*., 2003) recently demonstrated variable copy number indicative of gene duplication within the β–defensin cluster on Chr. 8p23. They showed that individuals can have from 2 to 12 copies of the region of DEFB4, DEF103, and DEFB104 encoding hBD-2, hBD-3, and hBD-4, respectively, per diploid genome. This repeat region does not include the genes encoding the α–defensins or hBD-1. Inheritance of genes encoding the α–defensins can also vary, but less extensively (Mars *et al*., 1995). The frequency of low vs. high copy number and the effect on peptide expression is not yet known, but may be an important factor in oral antimicrobial function.

Efficacy of antimicrobial peptides in the oral cavity

The oral cavity provides an inviting warm, moist environment conducive to colonization by many types of microorganisms. Thus the normal oral flora is extremely complex, requiring multiple types of defenses in order to prevent infection. These defenses include antimicrobial peptides with different, but overlapping, ranges of microorganisms against which they are effective. The oral cavity also provides an environment that allows optimal effectiveness of these peptides. Activity of most of the oral antimicrobial peptides is inhibited by ions/salt at physiologic levels (Goldman *et al*., 1997; Nagaoka *et al*., 2000; Turner *et al*., 1998); however, in the oral environment, they function at the surface away from high salt concentrations and interfering substances in the blood. Table 2 provides data from numerous sources on the activity of these peptides against various, mainly pathogenic, oral bacteria and fungi (see table for references for this section). The antimicrobial function of these peptides has been obtained through a myriad of methods, making comparisons and interpretations difficult. It should also be recognized that very few methods test for bactericidal (lethal) as opposed to bacteriostatic activity.

Peptide activity

LL-37. LL-37 is a cathelicidin with the conserved pro-region which keeps it inactive until proteases cleave this portion after the protein is secreted (Zanetti *et al*., 2000). Against oral microbes, LL-37 has the greatest activity against *A. actinomycetemcomitans* and *Capnocytophaga spp. A. actinomycetemcomitans* is an important pathogen associated with rapidly progressive forms of periodontal disease which often affect younger individuals. *Capnocytophaga spp.* have been implicated in juvenile gingivitis and periodontitis and can cause sepsis in immunocompromised patients (Gomez-Garces *et al*., 1994).

α-Defensins. α-Defensins are relatively ineffective against most of the oral microbes tested, with the exceptions of *Capnocytophaga ochracea* and *C. albicans*. However, synergy between HNP-1 and LL-37 has been demonstrated against *E. coli* and *Staphylococcus aureus* even at physiological salt concentrations (150 mM) (Nagaoka *et al*., 2000). HNP-1 requires target microbes to be metabolically active (Edgerton, *et al*., 2000). HNP-1–3 also show inhibitory effects on HIV infectivity.

β-Defensins. β-Defensins show both anti-fungal and anti-bacterial action. HBD-3 is consistently more active against both bacteria and fungi, with hBD-2 next and hBD-1 last in activity. The study by Joly and coworkers compared the effectiveness of hBD-2 and hBD-3 against oral microorganisms. HBD-2 and hBD-3 show considerable variability against multiple strains of the same species, but overall, aerobes are more susceptible than anaerobes. These peptides also show strain-specific activity toward various *Candida spp*. (Joly *et al*., 2004). Interestingly, Wu and coworkers showed that hBD-3 activity against *E. coli* was unaffected by disulfide bonding which was previously thought to be critical to antimicrobial function (Wu *et al*., 2003). Along with the α-defensins, hBD-2 and -3 inhibit HIV replication. HBD-2 has anti-fungal activity against *C. albicans* and *C. tropicalis,* while hBD-3 is active against other *Candida spp.* tested.

Histatins. Histatins are primarily anti-*Candida* agents. They have little effect on oral bacteria or on the fungus *Aspergillus fumigatus*. Histatin-5 is the most effective histatin and is active against *C. albicans* as well as other *Candida spp.* Histatins have activity in the concentration range found in saliva. Johnson, Yeh and Dodds (Johnson *et al*., 2000) measured histatin concentrations in saliva at 31 µg/ml in parotid secretions and 62 µg/ml in submandibular/submaxillary secretions. They also found that these concentrations decline by one half and one third, respectively, between the ages of 45 – 75. Synergistic effects have been demonstrated between histatin-5 and amphotericin B against *Candida spp.*, including an amphotericin B-resistant strain, *Cryptococcus neoformans* and *A. fumigatus* (van't Hof *et al*., 2000).

Table 2. Activity of antimicrobial peptides against oral microbes*								
Organism	hBD1	hBD2	hBD3	HNP-1	HNP-2/-3	LL-37	Histatin	Adrenomedulin
Escherichia coli[1,3,4,5,11,20,21,23]	40 (≥3); 5 (0.5)	10 (1); 0.6 (0.5)	5 (≥3); 0.1 (0.5)	50 (≥3)		1.0; 10 (≥3)		0.4
Streptococcus mutans[1,6,8,10,12]		4.1–7.8[b]	2.6–5[b]; 2 (≥3)				NS	12.5
S. sanguis[3,6,8,10]		8–25[b]	7.6–21.3[b]; 16 (≥3)			>100 (≥3)	NS	
S. salvarius[6]							NS	
S. sobrinus[10]			16 (≥3)					
Actinomyces naeslundii[1,6,8]		8–14[b]	4.1–7.2[b]				NS	12.5
Fusobacterium nucleatum[3,6,8,14]		6.3->250[b]	4.5->250[b]	>500		>100 (≥3)	NS	
Veillonella parvula[6]							(0.3)	
Prevotella intermedia[6]							NS	
Porphyromonas gingivalis[1,3,8,10,12]		34.6->250[b]	5.7->250[b]; 100 (≥3)			>100 (≥3)		$7.75x10^{-4}$
Actinobacillus actinomycetemcomitans[3,8,10,12,13,15]		>250	9.6->250[b]; 2.5 (≥3)	NS (1)	NS (1)	10 (2); >100 (≥3)		
Lactobacillus acidophilus[10]			8 (≥3)					
Capnocytophaga sputigena[13,19]				200 (1); 500 (2)	500 (1)	7.5 (2)		
C. gingivalis[13,19]				200 (1)	500 (1)	9.1 (2)		
C. ochracea[13,19]				10 (1); 500 (2)	10 (1)	11.0 (2)		
Candida albicans[1,2,3,7,8,9,17,18,20]	>500	25 (1); 125; 4.6–59[b]	25;2.8–7.1[b]	>250 (≥3); 96% (52 μg/ml) LOV		>250 (≥3); >100 (≥3)	97% (14 ug/ml) LOV; >500 [24 hr]; 2.4 (0.5); 50 μM (1.5); 100 μM (2)	NS
resist. *C. albicans*[9,22]		125	25				125 [24 hr]	
C. glabrata[7,8,9,15,22]		23->250[b]; >1000	34->250; 50				62.5 [24 hr]; 29 (0.5); 42–87% LOV[a]	
resist. *C. glabrata*[7,9]		>1000	200				1.0 (0.5)	
C. krusei[3,7,8,9,15,22]		12->250[b]; >1000	2–13.7[b]; 200			>100 (≥3)	500 [24 hr]; 1.2 (0.5); 80–95% LOV[a]	
C. neoformans[7,22]							31.3 [24 hr]; 0.7 (0.5)	
C. pseudotropicalis[7]							1.0 (0.5)	
C. parapsilosis[7,8,9,15]		9.3–17.8[b]; >1000	1.4–12.4[b]; 50–100				2.0[e]; 72–98% LOV[a]	
C. tropicalis[3,8,9,15]	>500	3.9–13.1[b]; 125	3.3–14.4[b]; 6.25			>100 (≥3)	90–98% LOV[a]	
C. guilleirmondii[15]							90–98% LOV[a]	
Aspergillus fumigatus[22]							>500 [24 hr]	
HIV[15,16,24]	NO	YES	YES	YES	YES			
*Values in MIC (μg/ml) unless otherwise noted.								

Table 2. Key		
Key	References	
	[1]Allaker & Kapas, 2003	[13]Miyasaki *et al*., 1990
(#) = # log reduction	[2]Edgerton *et al*., 2000	[14]Miyasaki & Lehrer, 1998
	[3]Guthmiller *et al*., 2001	[15]Nikawa *et al*., 2001
[24 hr] = read at 24 hr	[4]Harder *et al*., 2001	[16]Quinones-Mateu *et al*., 2003
	[5]Harder *et al*., 1997	[17]Raj *et al*., 1990
LOV = loss of viability	[6]Helmerhorst *et al*., 1999a	[18]Schröder & Harder, 1999
	[7]Helmerhorst *et al*., 1999b	[19]Tanaka, Miyasaki & Lehrer, 2000
NS = no significant activity	[8]Joly *et al*., 2004	[20]Turner *et al*., 1998
	[9]Jurevic, 2004	[21]Valore *et al*., 1998
[a] range due to different strains (50 μM)	[10]Maisetta *et al*., 2003	[22]Van't Hof *et al*., 2000
	[11]Mandal & Nagaraj, 2002	[23]Wu *et al*., 2003
[b] MIC range due to strain differences	[12]Mineshiba *et al*., 2003	[24]Zang *et al*., 2002

Antimicrobial peptides as therapeutic agents in the oral cavity

Antimicrobial peptides are under investigation for control of oral infections. One such drug is Iseganan, an analog of protegrin-1, a porcine cathelicidin, which has broad spectrum bactericidal activity. Iseganan has recently completed Phase III clinical trials for prevention of ulcerative oral mucositis. It showed encouraging results in reducing the occurrence of oral mucositis and associated clinical problems such as mouth pain, throat pain and difficulty swallowing (Giles *et al*., 2003). A second investigational peptide is the antifungal, histatin-5. The yeast *Candida* is a commensal organism on skin and mucosal surfaces but is a growing problem in causing opportunistic infections. Frequency of mucosal and invasive fungal infections has increased in association with increased numbers of immunocompromised patients due to HIV, chemotherapy, and the use of immunosuppressive drugs. In addition, there has been an increase in *Candida* strains that are resistant to the main therapeutic drugs in current use. This has led to an increased interest in new antifungals, primarily antifungal peptides. Histatin-5 and several variants are under active investigation for this purpose since their mechanism of action differs from the main antifungal drugs in current use and they are effective against azole-resistant *Candida* strains (Situ and Bobek, 2000; Tsai and Bobek, 1998).

Conclusions

The oral cavity is a unique environment in which antimicrobial peptides play a key role in maintaining health. Present evidence suggests that α–defensins, β–defensins, LL-37, histatin, and other antimicrobial peptides and proteins have distinct but overlapping roles in maintaining oral health. The expression of the inducible hBD-2 in normal oral epithelium, and the apparent differential signaling in response to commensal and pathogenic organisms, provides new insights into innate immunity in this body site. Commensal bacteria are excellent inducers of hBD-2 in oral epithelial cells, suggesting that the commensal bacterial community acts in a manner to benefit the overall innate immune readiness of oral epithelia. This may have major significance for understanding host defense in the complex oral environment.

Future trends

Future studies will emphasize approaches to learn more about the role of antimicrobial peptides in oral health and susceptibility to disease and infection. Because periodontal disease is largely a situation in which the inflammatory process has gone awry, it is critical to understand the role of innate immunity in the recognition of, response to, and effectiveness against oral microbes. This includes more detailed information about both α– and β–defensins, LL-37, and other oral antimicrobial peptides and proteins. These peptides very likely have specific functions in keeping the level of commensal bacteria in check as well as being effective against pathogens. The involvement of antimicrobial peptides in other mucosal disorders is also important to understand. In oral squamous cell carcinoma and lichen planus, β–defensin expression may reveal clues on disease progression and prognosis. Recent evidence of the involvement of β–defensins in the defense against HIV infection and transmission in the oral environment is fascinating (Quinones-Mateu *et al*., 2003). There is much more to learn about this effect and how it may be useful therapeutically. Further, studies of expression and function of the newly described members of the β–defensin family (Schutte *et al*., 2002) may provide critical new pieces of information relating to oral health.

The initial genetic studies suggesting a protective role of an hBD-1 SNP need extension and confirmation. This finding opens a whole new area of investigation. Does the hBD-1 SNP affect *Candida* carriage directly via hBD-1 or indirectly via a linked gene? Can this SNP be used as part of a diagnostic susceptibility profile in situations in which *Candida* infection could become life threatening, such as transplant recipients? Are defensin SNPs correlated with other oral infections? Because defensins are secreted and may function synergistically with salivary antimicrobials they may protect against dental caries, or conversely contribute to genetic susceptibility to dental decay. Similar questions can be asked of the role of LL-37 in oral health and disease. Genetic investigations could be extended to include molecular mechanisms and defects in processing

of antimicrobial peptides in neutrophils, such as in Morbus Kostmann syndrome and possibly in Papillon-Lefevre syndrome in view of the profound effects of neutrophil antimicrobials on periodontal health.

Another major area of future study is the regulation by and functional efficacy against oral commensal vs. pathogenic organisms. Present evidence suggests that innate immune responses of oral epithelia are primed by commensal bacteria and this occurs via pathways other than those utilized in response to pathogens. This ability of epithelial cells to distinguish commensal and pathogens may represent a general phenomonen since epithelia from different body sites co-exist with different populations of commensal bacteria. Study of the regulation of β-defensins is a window opening our understanding of this important aspect of innate immunity of oral epithelia as well as epithelia in other body sites. It will also be important to understand the molecular mechanism by which the defensins and LL-37 exert their multiple functions in promoting wound healing and linking innate immunity to acquired immunity.

Finally, the development of new peptide antimicrobial agents for therapeutic use in the oral environment is an important area for future investigation and testing. Peptide antimicrobial agents may augment the innate defenses of individuals at high risk for oral infection because of compromised immune status.

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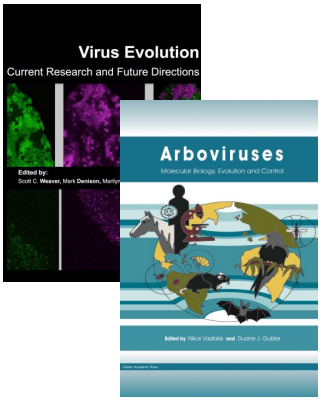

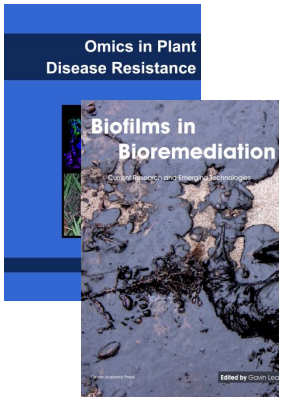

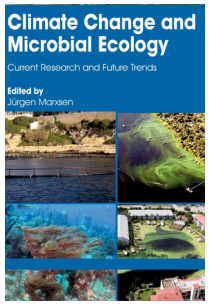

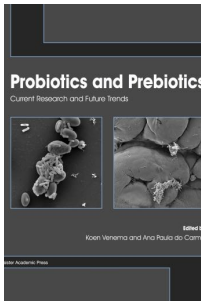

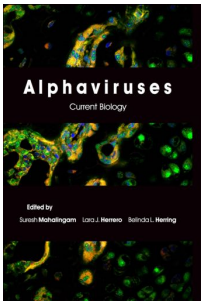

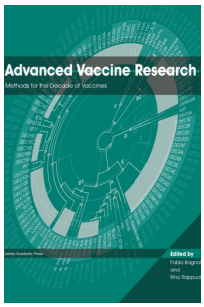

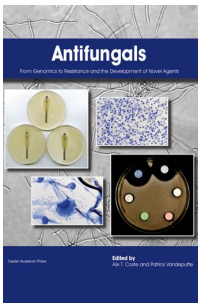

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