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Review

Antimicrobial peptides in defence of the oral and respiratory tracts

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Abstract

Antimicrobial peptides (AMPs) are components of complex host secretions, acting synergistically with other innate defence molecules to combat infection and control resident microbial populations throughout the oral cavity and respiratory tract. AMPs are directly antimicrobial, bind lipopolysaccharide (LPS) and lipoteichoic acid, and are immunomodulatory signals. Pathogenic and commensal organisms display a variety of resistance mechanisms, which are related to structure of cell wall components (e.g. LPS) and cytoplasmic membranes, and peptide breakdown mechanisms. For example, LPS of the AMP-resistant cystic fibrosis pathogen *Burkholderia cepacia* is under-phosphorylated and highly substituted with charge-neutralising 4-deoxy-4-aminoarabinose. Additionally, host mimicry by addition of phosphorylcholine contributes to resistance in oral and respiratory organisms. *Porphyromonas gingivalis*, *Pseudomonas aeruginosa* and other pathogens produce extracellular and membrane-bound proteases that degrade AMPs. Many of these bacterial properties are environmentally regulated. Their modulation in response to host defences and inflammation can result in altered sensitivity to AMPs, and may additionally change other host–microbe interactions, e.g. binding to Toll-like receptors. The diversity and breadth of antimicrobial cover and immunomodulatory function provided by AMPs is central to the ability of a host to respond to the diverse and highly adaptable organisms colonising oral and respiratory mucosa.

Keywords: Antimicrobial peptides; Defensins; LL-37; Innate defences; *Porphyromonas gingivalis*; *Burkholderia cepacia*; *Pseudomonas aeruginosa*; Periodontal disease; Cystic fibrosis; Proteases; Lipopolysaccharide

1. Introduction

Cationic antimicrobial peptides (AMPs) have emerged as central components of mammalian innate defences and are of fundamental relevance to understanding host–microbe relationships. The importance of AMPs extends beyond their direct antimicrobial activity, as their broad biological activities indicate they are effector molecules, providing communication between innate and adaptive immune systems (Yang et al., 2002). An important property of AMPs is their ability to bind avidly to many potentially pro-inflammatory molecules released from micro-organisms, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA) and DNA. By binding to these molecules, AMPs inhibit responses of host cells and damp-down an undesirable inflammatory response (Scott et al., 1999, 2000a,b; Nagaoka et al., 2001). This may be a key function, in which AMPs function alongside or in concert with other LPS binding molecules, such as lipopolysaccharide binding protein (LBP) and bactericidal permeability inducing protein (BPI), to regulate immune responses at mucosal sites assaulted by large numbers of bacteria and, in particular, their released cellular components.

AMPs are synthesised within granules of phagocytic cells or are secreted by epithelia. At each site of production, they form part of a cocktail of antimicrobial substances which in vivo work synergistically to combat infection (Gudmundsson and Agerberth, 1999; Hancock and Diamond, 2000). Their tissue specific expression is also likely to be a significant contributor to the tissue tropism displayed by pathogenic and resident micro-organisms. AMPs have evolved in response to the positive selection pressures exerted by colonising micro-organisms (Hughes, 1999), and pathogens and commensals alike have developed strategies for surviving or evading the activities of AMPs (Devine and Hancock, 2002). In humans and other mammals, some sites are usually free of micro-organisms (e.g. lung), whilst others (e.g. oral cavity) are heavily colonised by diverse populations. The oral cavity and respiratory tract are similar in the range of AMPs they express, and increasing attention has been paid to the roles of AMPs at these sites in defence against conditions such as periodontal disease and cystic fibrosis (CF) associated lung disease. AMPs

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are additionally important as potential novel anti-infective agents for treatment of such diseases.

2. Antimicrobial peptides in oral innate defences

2.1. Production

Tissues of the oral cavity are constantly exposed to innate defence components (Table 1) derived from saliva, gingival crevicular fluid and epithelial cells (Dale et al., 2001; Lamont and Jenkinson, 2000; Marsh, 2003). Saliva contains a range of innate defence molecules that are either directly antimicrobial or interfere with microbial colonisation or nutrition. Many of these molecules function synergistically and some, such as the lysozyme–protease system and histatins (histidine-rich AMPs), are potentiated by acid, which may be particularly relevant in defence against dental caries. AMPs are important components of these oral innate defences and a number are secreted by salivary glands and epithelial cells and are released from neutrophils.

Histatins are secreted by salivary glands, following transcription from two genes. Subsequent proteolysis of the primary translation products results in at least 12 forms of salivary histatin, only some of which are antimicrobial (Xu et al., 1991). Histatins are mainly anti-candidal, although they have been shown to inhibit some Gram-positive bacteria and the periodontal pathogen *Porphyromonas gingivalis* (Murakami et al., 1991).

Like most human epithelia, oral epithelia are protected by production of β-defensins (HBDs). HBD1 is expressed constitutively in salivary glands, gingiva, buccal mucosa and tongue (Zhao et al., 1996; Krisanaprakornkit et al., 1998; Bonass et al., 1999; Mathews et al., 1999; Sahasrebudhe et al., 2000). In saliva, β-defensins are associated with mucin (Sahasrebudhe et al., 2000), which may protect them from degradation and could also increase their contact with mucin-aggregated bacteria. Such binding may also facilitate concerted activities with other mucin-associated molecules synthesised by salivary glands, such as the trefoil factor family (TFF) proteins (Devine et al., 2000). TFFs are wound healing motogenic proteins involved in early restitution of damaged epithelia that also bind bacteria (dos Santos Silva et al., 2000). Isoforms of HBD1 have been detected in oral epithelial cell culture supernatants (Diamond et al., 2001). Isoforms of HBD1 and HBD2 are produced at other sites and in many mammalian species. This may provide added breadth of antimicrobial cover, as minor differences in amino acid sequence can produce significant differences in defensin antimicrobial activity (Raj et al., 2000; Devine and Hancock, 2002). Inducible expression of HBD2 and HBD3 have also been observed in oral epithelial cells and salivary glands (Bonass et al., 1999; Mathews et al., 1999; Jia et al., 2000; Krisanaprakornkit et al., 1998; Garcia et al., 2001a; Dunsche et al., 2002), although each peptide is induced through different signalling pathways (discussed later). Another putative antimicrobial peptide with a defensin-like 6-cysteine motif, HE2β1, is expressed in human gingival epithelia (Jia et al., 2001).

Phagocytic cells are significant sources of AMPs. Neutrophils are the richest and best-studied sources, but macrophages, monocytes and dendritic cells also synthesise HBD1 and HBD2 (Duits et al., 2002) and may be sources of these AMPs at a variety of sites. Neutrophil AMPs are found throughout the oral cavity, and increase in concentration following inflammation. α-Defensins HNP1–4 have been detected in saliva, gingival crevicular fluid and in gingival junctional epithelium (McKay et al., 1999; Mizukawa et al., 1999; Dale et al., 2001). The human cathelicidin LL-37 is produced within secondary granules of neutrophils, but is also secreted by epithelial cells at a wide range of sites following induction by microbial products or inflammatory mediators. Its secretion (or that of its precursor hCAP18) has been detected in tongue and buccal mucosa (Frohm Nilsson et al., 1999) but LL-37 peptide detected in gingival junctional epithelium was most likely derived from neutrophil infiltration (Dale et al., 2001). Whilst we have detected LL-37 peptide in salivary gland ducts, expression appeared to be low compared with HBD1 (Fig. 1).

Other multifunctional molecules with AMP properties or activities are also found within oral tissues and fluids. Adrenomedullin is antimicrobial as well as vasodilatory and it is induced by exposure of oral epithelial cells to bacteria but not by *Candida albicans* (Kapas et al., 2001). Some cationic fragments derived from larger proteins exhibit AMP functions; for example, fragments of lactoferrin, bactericidal permeability inducing peptide, histones, ribosomal protein, haemoglobin and mucin (Devine and Hancock, 2002; Bobek and Situ, 2003).

Table 1
Innate defence molecules in oral fluids and secretions

Salivary components	Gingival crevicular fluid components	Epithelial cell secretions
Histatins	α-Defensins (HNP1–4)	α-Defensins (HNP1–4)
α-Defensins (HNP1–4)	β-Defensins (HBD1–3)	β-Defensins (HBD1–3)
β-Defensins (HBD1–3)	LL-37	HE2β1
LL-37	IgG	LL-37
Secretory IgA	IgA	IgA
Mucins	IgM	Calprotectin
Lysozyme	Complement	
Protease		
Lactoferrin		
Sialoperoxidase		
Proline-rich proteins		
Statherin		
Fibronectin		
Cystatins		
Trefoil factor family proteins		

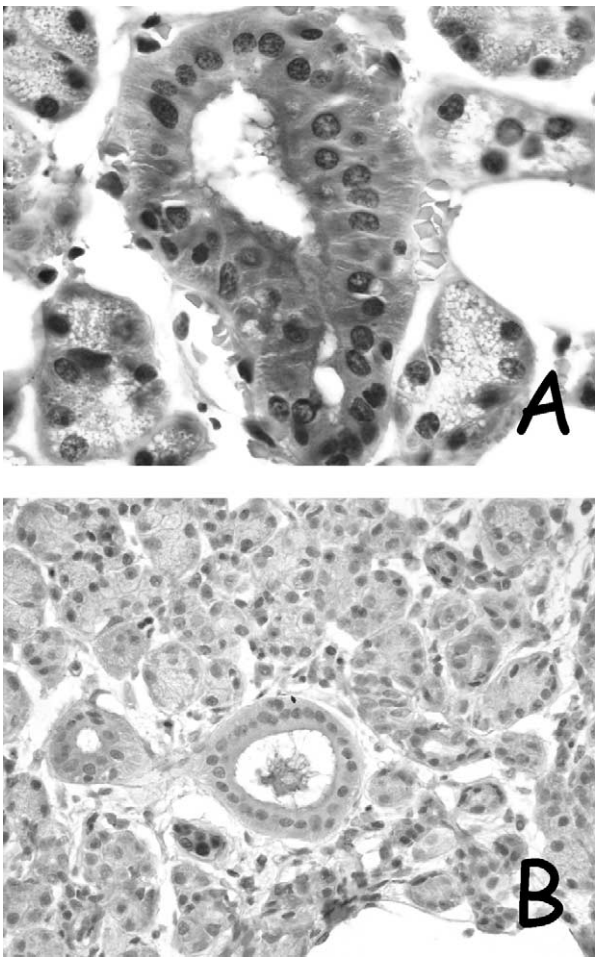

Fig. 1. Immunostaining of human submandibular gland with antibodies to β-defensin 1 (A) and LL-37 (B).

2.2. Roles of antimicrobial peptides in oral innate defences

The micro-organisms encountered by AMPs and other oral innate defence molecules are numerous and diverse. Close or adjacent sites harbour distinct microbiota; oral microbiota differ significantly from upper respiratory tract populations despite their proximity and, indeed, there are variations even within the oral cavity (Hohwy et al., 2001; Rasmussen et al., 2000; Könönen et al., 2002; Marsh, 2003). It is estimated that up to 600 species, only 50% of which can be grown in monoculture by conventional methods, are normal inhabitants of the human mouth (Wilson et al., 1997; Paster et al., 2001). These populations exhibit considerable diversity and inter-dependent consortia, rather than individual organisms, are associated with diseases (Marsh, 2003). In such cases, it can be difficult to determine which organisms contribute to the aetiology of the disease and which are bystanders in the process. Nonetheless, certain organisms are consistently implicated in the aetiology of advanced periodontal diseases, for example, the Gram-negative anaerobe *P. gingivalis*, which is isolated in low numbers from healthy subgingival sites but increases in prevalence during disease. It produces a range of virulence determinants, including potent proteases, adhesins, LPS and haemagglutinins (Lamont and Jenkinson, 2000) and is able to survive and grow within oral epithelial cells (Houalet-Jeanne et al., 2001; Rudney et al., 2001).

Proving or examining the roles of AMPs in defence of the oral cavity is particularly challenging, because of the complexity of the microbiota and the multiplicity of innate defence molecules produced. A study of a congenital condition associated with severe neutropenia linked periodontal disease with a deficiency of neutrophil AMPs (Putsep et al., 2002). Epithelial AMPs are more numerous (Schutte and McCray Jr., 2002) and greater overlap or redundancy of function is likely, as is indicated by the fact that BD1-deficient mice showed no overt signs of ill health or infection (Morrison et al., 2002; Moser et al., 2002). This may be particularly important in defence of heavily colonised sites like the mouth, where loss of adequate control of such resident populations could be catastrophic. At these sites AMPs not only exhibit a breadth of recognition to accommodate microbial diversity, but also provide sufficient redundancy to ensure such populations are controlled, avoiding disruption of host–microbe homeostatic mechanisms.

Exposure of oral epithelial cells to a range of bacterial products, such as LPS, and inflammatory mediators induce synthesis of β-defensins. Few studies have examined induction by organisms that are directly relevant to the oral cavity. However, Krisnaprakornkit et al. (2000) showed that cell wall extracts of *Fusobacterium nucleatum* induced gingival epithelial cells to synthesise HBD2, while extracts of *P. gingivalis* did not. This may or may not be related to the ability of these two organisms to cause disease. Whilst *P. gingivalis* is generally recognised as a periodontal pathogen and increases in prevalence with disease, *F. nucleatum* is isolated with equal frequency from healthy and diseased sites but has been associated by some groups with periodontal disease and additionally demonstrates properties associated with pathogenicity (Haffajee et al., 1999; Han et al., 2000). To date, a clear cut relationship between pathogenicity or commensalism and interactions with AMPs has not emerged, and bacteria employ a range of strategies for surviving AMPs (Devine and Hancock, 2002).

Histatins have been somewhat overlooked in many discussions of AMPs, possibly because they appear to be specific to the mouth. This may indicate that their spectrum of activity, particularly against *Candida* spp., is of primary importance to defence of the oral cavity. It may also reflect a need to protect critical cells, for example, secretory cells in salivary ducts, although AMPs are probably not essential for preventing colonisation of other protected sites such as intestinal crypts (Garabedian et al., 1997). *Candida* spp. frequently colonise oral mucosal surfaces, causing disease in immunocompromised or antibiotic treated individuals. Reduced salivary flow and lower salivary histatin concentrations have been linked with *Candida* colonisation of oral

mucosa (Jainkittivong et al., 1998). Whether this was a reflection of the ability of *Candida* spp. to repress secretion of histatins was not explored. *C. albicans* did not induce oral keratinocytes to up-regulate adrenomedullin production, in contrast to Gram-positive and Gram-negative bacteria (Kapas et al., 2001). The role in oral ecology of histatins may be greater than their principally anti-candidal activities indicate since they also: (i) inhibit *P. gingivalis* and host proteases (Gusman et al., 2001); (ii) inhibit *P. gingivalis* adhesion to erythrocytes and streptococci (Murakami et al., 1991, 1992); (iii) suppress induction of cytokines by *P. gingivalis* outer membrane proteins (Imatani et al., 2000).

3. Antimicrobial peptides in respiratory innate defences

3.1. Production

As in the mouth, the airways are protected by AMPs released from phagocytes and secreted by epithelial cells. Neutrophil α-defensins HNP1–4 and cathelicidin LL-37, derived from neutrophils and epithelial cells, are detected in airway secretions (Bals et al., 1998b; Agerberth et al., 1999). Airway epithelial cells secrete α-defensin HD5 and β-defensins HNP1–4 (McCray Jr. and Bentley, 1997; Goldman et al., 1997; Bals et al., 1998a; Singh et al., 1998; Frye et al., 2000; Jia et al., 2001; Harder et al., 2001; Garcia et al., 2001a,b). HBD2 and LL-37 have been shown to reach significant levels in bronchial alveolar lavage fluid (Singh et al., 1998; Agerberth et al., 1999). While HBD1 is expressed constitutively, the other β-defensins are inducible but not by the same stimuli. Unlike HBD2, the gene encoding HBD3 does not have a NFκB consensus sequence but does have AP1 and NF–IL-6 consensus sequences, and the *HBD3* gene is up-regulated by IFN-γ, not TNF-α, IL-1α, IL-6, PMA or non-viable *Pseudomonas aeruginosa* (Garcia et al., 2001a). HBD4 is primarily expressed in the lung and is induced by non-viable *P. aeruginosa* and *Streptococcus pneumoniae* but not by TNF-α, IFN-γ or IL-1β (Garcia et al., 2001b). *P. aeruginosa* is a significant respiratory pathogen, and is particularly associated with high morbidity and mortality in patients suffering from CF. It has been shown to induce β-defensin synthesis in mouse respiratory epithelia in vivo (Bals et al., 1999a; Morrison et al., 1999). The production of mucoid extracellular polysaccharide by *P. aeruginosa* has been linked to virulence and Harder et al. (2000) found a mucoid *P. aeruginosa* strain induced HBD2 production in respiratory epithelial cells but non-mucoid strains did not. This was true for cells derived from CF patients as well as non-CF individuals. However, CF patients did not up-regulate β-defensins in response to inflammatory mediators (Dauletbaev et al., 2002), supporting a contention that local deficiency in innate defences is important in the pathogenesis of CF lung disease (Bals et al., 1998a).

In the respiratory tract, as in the oral cavity, AMPs form part of a cocktail of antimicrobial molecules (Table 2) many of which increase after infection and inflammation (Diamond et al., 2000; Zhang et al., 2000; Schutte and McCray Jr., 2002). Synergy has been demonstrated between BPI and phospholipase A2, HBD2 and lactoferrin, as well as between HBD2/HBD4 and lysozyme (Bals et al., 1998a; Garcia et al., 2001b). However, other studies did not confirm synergy between HBD2, HBD3 or HBD4 and lysozyme (Singh et al., 2000; Garcia et al., 2001a) so this needs further clarification. Defensins and many other AMPs are inhibited in vitro by increasing concentrations of NaCl, but synergy between HNP1 and LL-37 overcame the inhibitory effects of NaCl (Nagoaka et al., 2000). AMPs are one of many LPS binding molecules produced in the respiratory tract, including BPI, LBP and surfactant-associated proteins (Crouch et al., 2000; Zhang et al., 2000; Augusto et al., 2002). α-Defensins regulate release of serine proteinase inhibitor (SLPI) from airway epithelial cells (van Wetering et al., 2000). Elafin, which like SLPI is an inhibitor of neutrophil proteinase activity, is also directly antimicrobial (Simpson et al., 1999) and is regulated by neutrophil defensins (van Wetering et al., 2000).

Table 2
Innate defence molecules in airway surface fluid

Antimicrobial component	Produced by cells
α-Defensins (HNP1–4)	Neutrophils
α-Defensin (HD5)	Epithelial
β-Defensins (HBD1–4)	Epithelial, macrophages, monocytes, dendritic
LL-37	Neutrophils, epithelial
Lysozyme	Epithelial, neutrophils
Phospholipase A2	Epithelial, neutrophils
IgA	Epithelial
Lactoferrin	Epithelial, neutrophils
Bactericidal permeability inducing protein	Neutrophils
Serine proteinase inhibitor	Epithelial, macrophages
Surfactant proteins SP-A, SP-D	Epithelial
Anionic peptides	Epithelial
Proline-rich proteins	Epithelial
Trefoil factor family proteins	Epithelial

3.2. Roles of antimicrobial peptides in respiratory innate defences

Inducible and constitutive production of AMPs is known to occur throughout the respiratory tract and levels of β-defensins and LL-37 increase following infections and inflammation (Ashitani et al., 2001; Dauletbaev et al., 2002; Lee et al., 2002; Schaller-Bals et al., 2002). They protect sites that are heavily colonised, such as the nasal cavity and nasopharynx, and some which are usually free of micro-organisms, e.g. the lung. The diverse resident commensal populations of the upper airways consist of organisms including *Staphylococcus* spp., *Streptococcus* spp., *Neisseria* spp., *Moraxella* spp., *Haemophilus* spp. and *Mycoplasma* spp. Some respiratory pathogens

do not cause clinically overt disease in all hosts, and important pathogens may be carried as part of the normal resident microbiota of individuals for considerable periods of time. Some opportunistic pathogens cause severe infections in patients with CF. In addition to *P. aeruginosa*, members of the *Burkholderia cepacia* complex are important causes of morbidity and mortality in this group (Mohr et al., 2001; Mahenthiralingam et al., 2002). Up to 20% of patients with CF who acquire *B. cepacia* complex organisms develop the "cepacia syndrome", a rapidly fatal necrotising pneumonia, often in conjunction with septicaemia. Virulence and clinical outcome are correlated to specific *B. cepacia* genomovars, many of which have been assigned to new species (Mahenthiralingam et al., 2002).

The virulence determinants of *B. cepacia* complex organisms have received a great deal of attention in recent years but pathogenicity is not yet fully understood. Adhesion and colonisation of respiratory epithelia are important, and in pulmonary infection *B. cepacia* may exhibit a biofilm mode of growth (Desai et al., 1998). Colonisation may be aided by the fact that these bacteria are highly resistant to a wide range of human and non-human AMPs. In our studies, strains representing six genomovars were resistant to HNP1, HBD1, HBD2 and LL-37 and other non-human AMPs (Table 3). However, many were sensitive to ovine cathelicidin SMAP-29 and the synthetic D2A-22, both of which have been reported previously to have activity against selected strains of *B. cepacia* (Schwab et al., 1999; Saiman et al., 2001). Whilst virulence is correlated with particular genomovars of the *B. cepacia* complex, there was no clear relationship between sensitivity to these peptides and genomovar. Differences in outer membrane structure amongst these strains are currently being examined.

Table 3
Resistance of *B. cepacia* complex strains representing genomovars I–VI[a] to antimicrobial peptides

Peptide	Number resistant (number studied)	MCZ[b] of sensitive strains	MIC[c] of sensitive strains
β-Defensin 1	17 (17)	>500	>500
β-Defensin 2	17 (17)	>500	>500
LL-37	17 (17)	>500	>500
HNP1	17 (17)	>500	>500
D2A-22	6 (14)	4–250	0.5–250
SMAP-29	4 (13)	4–250	0.5 to >500
Histatin Dhvar4	14 (14)	>500	>500
Brevinin 1	16 (16)	>500	>500
Cecropin B	12 (13)	62	0.5
Melittin	16 (17)	1	0.5
Polymyxin B	17 (17)	>500	>500

[a] Genomovars II, IV and V have been assigned to *Burkholderia multivorans*, *Burkholderia stabilis* and *Burkholderia vietnamensis*, respectively (Mahenthiralingam et al., 2002).

[b] Minimum concentration of peptide ($\mu g\,ml^{-1}$) producing a zone of inhibition in double layer agarose assays.

[c] Minimum inhibitory concentration ($\mu g\,ml^{-1}$) determined in broth microdilution assays.

An important role for AMPs in the susceptibility of the CF lung to infection has been suggested. It was proposed that, as a result of the CF defect in ion transport, tracheal exudates of CF patients have high concentrations of NaCl, causing inactivity of AMPs towards pathogenic bacteria and thereby contributing to the ability of the latter to infect these patients (Goldman et al., 1997). However, not all studies have confirmed high NaCl concentrations in airway fluids and it is a paradox that organisms like *B. cepacia*, which are naturally resistant to AMPs, should require salt-inactivation of these innate defences to express their pathogenicity. Also, HBD3 was recently shown to kill *B. cepacia* regardless of NaCl concentration (Garcia et al., 2001a). It is likely that multiple factors are in operation. It has been suggested that more general, uncharacterised, deficiencies in local innate defence of the CF lung are responsible for the increased susceptibility to infection, and AMPs may be a component of this. *B. cepacia* can survive within respiratory epithelial cells, macrophages and in amoeba and this may be related to AMP resistance as well as other factors, e.g. a decrease in NO production in *B. cepacia* infected macrophages has been reported as well as cytotoxicity to macrophages (Mohr et al., 2001).

A number of in vivo experimental models have been used to determine the functional importance of AMPs in defence of the respiratory tract, particularly in CF. Over-expression of the human peptide LL-37 in a CF mouse model resulted in increased killing of *P. aeruginosa* (Bals et al., 1999c), reduced ability of *P. aeruginosa* to colonise the lung epithelium and in reduced inflammation and susceptibility to septic shock (Bals et al., 1999b). Mice deficient in mBD1 expression did not show any overt signs of ill health or abnormality (Morrison et al., 2002; Moser et al., 2002). These studies indicated that an individual AMP may be more important in defence against one organism than another. For example, mBD1-deficient mice were inefficient at clearing *Haemophilus influenzae* from lungs and airways (Moser et al., 2002) but eliminated *Staphylococcus aureus* from their lungs as efficiently as wild type mice (Morrison et al., 2002), while mutant and wild type mice were equally susceptible to infection, sepsis and death following infection with *S. pneumoniae* (Moser et al., 2002).

In addition to defending against infection, released α-defensins may contribute to epithelial repair in the respiratory tract through enhancing lung epithelial cell proliferation (Aarbiou et al., 2002). To this end, they may function alongside wound healing factors such as the TFF proteins, which are also expressed by respiratory epithelia (dos Santos Silva et al., 2000). AMPs also contribute to protection against protease-mediated damage through regulation of release of SLPI and elafin. On the other hand, neutrophil AMP release can also have undesirable effects (van Wetering et al., 1999; Devine and Hancock, 2002) and

may contribute to the pathogenesis of certain respiratory diseases, such as inflammatory lung disease and atherosclerosis (through binding of lipoproteins). α-Defensins adhere to indwelling medical devices and diminish effective defences against biofilm formation by inhibiting neutrophil function, and these AMPs can also enhance the adhesion of respiratory pathogens to respiratory epithelial cells.

Thus, AMPs are components of complex host secretions contributing to innate defences throughout the oral cavity and respiratory tract. They act synergistically with each other and with other classes of molecule to combat infection and control resident microbial populations. They may function alongside molecules such as TFF proteins in wound healing processes, and with other LPS binding molecules such as bactericidal permeability inducing protein and LPS binding protein to regulate responses to bacterial LPS.

4. Mechanisms of antimicrobial peptide resistance displayed by oral and respiratory micro-organisms

In many cases antimicrobial peptides are able to kill bacteria by depolarising and permeabilising membranes (Fig. 2), but there are some examples in which the lethal target is cytoplasmic (reviewed in Devine and Hancock, 2002). In Gram-negative bacteria, peptides first associate with negatively charged moieties of the outer membrane, producing structural cracks. They also bind to the divalent cation-binding sites of polyanionic surface LPS and expand the outer membrane by displacing divalent cations, which normally stabilise outer membrane structure. Disruption of barrier function and integrity of the outer membrane then allows passage of molecules such as large hydrophobic antibiotics and the AMPs themselves (termed the self-promoted uptake pathway; Hancock and Chapple, 1999). AMPs then bind to the interfacial region of the cytoplasmic membrane

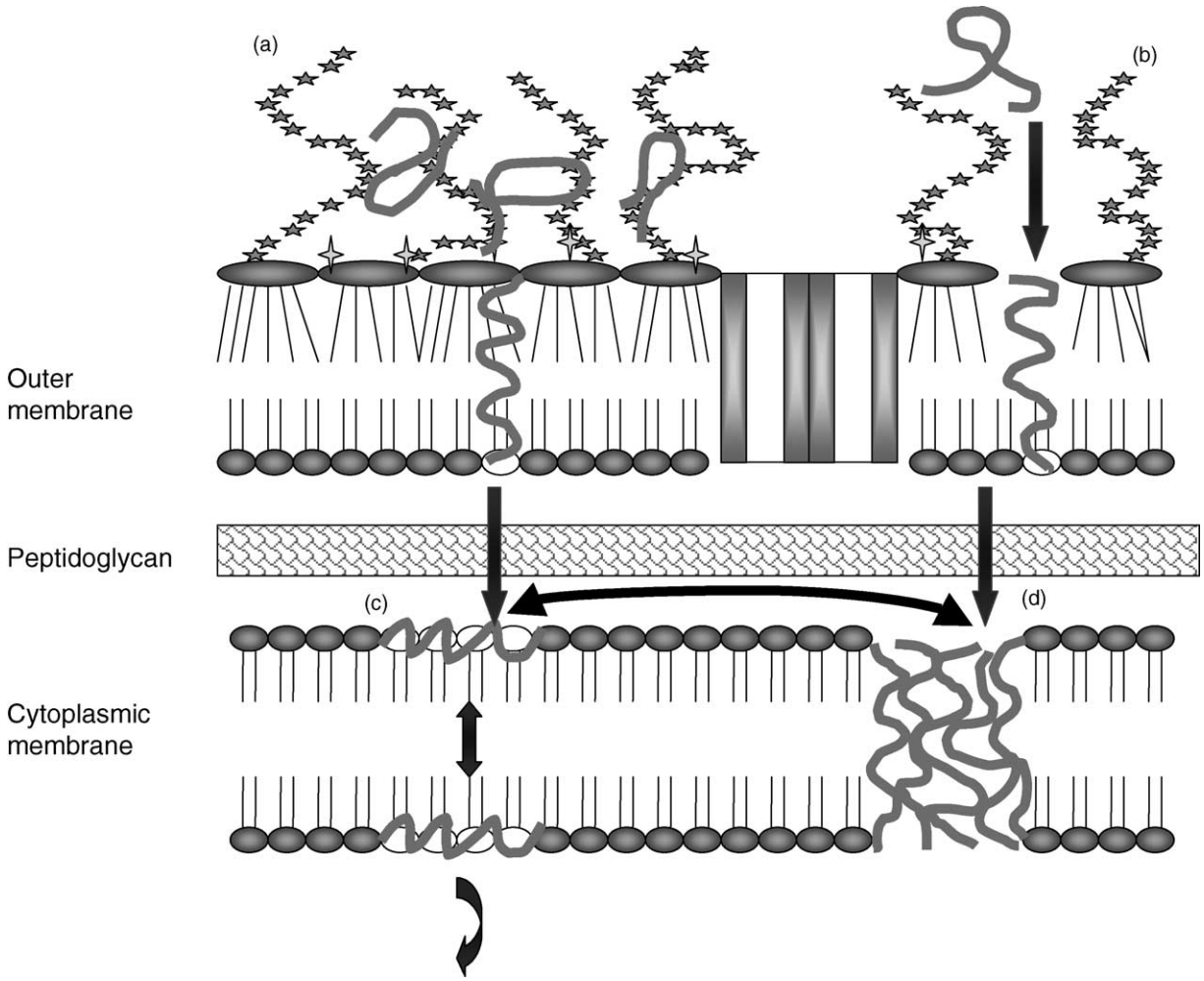

Fig. 2. Interactions between antimicrobial peptides and Gram-negative bacterial cells (adapted from Hancock and Chapple, 1999). (a and b) Peptides bind to the divalent cation-binding sites and associate with the polyanionic outer moieties of LPS, disrupting and expanding the outer membrane and allowing passage of AMPs through the outer membrane. (c) AMPs then bind to the interfacial region of the cytoplasmic membrane. (d) When at sufficient concentrations, AMPs aggregate within the membrane causing depolarisation and permeabilisation. Some monomers may detach and gain access to the cytoplasm.

and, as they reach sufficient concentrations, aggregate within the membrane. Gram-positive bacteria do not have an outer membrane, but AMP binding to outer wall components such as lipoteichoic acids nonetheless play a role in the mechanism of action, as changes to these molecules affect sensitivity to AMP killing (discussed later).

Resistance of bacteria to killing by peptides is determined by a number of bacterial properties including charge density and structure of outer wall components such as LPS, lipid composition of the cytoplasmic membrane, the presence of an electrochemical potential across the cytoplasmic membrane, responses of bacterial cells to environmental changes and stresses, and peptide breakdown, transport and efflux mechanisms (Devine and Hancock, 2002; Peschel, 2002). Some of these mechanisms are particularly relevant to oral and respiratory organisms.

4.1. Lipopolysaccharide

Bacterial LPS is an important virulence determinant for many Gram-negative pathogens and it exhibits a number of important properties, such as immunogenicity, induction of pro-inflammatory cytokines, and protection against phagocytosis and complement killing. LPS consists of three components: lipid A (which anchors the molecule in the outer membrane) is linked to the 3-deoxy-α-D-manno-oct-2-ulopyranosonic acid (Kdo) of the core oligosaccharide, which is in turn linked to the outer component of LPS, the O-polysaccharide. Whilst the O-polysaccharide is highly variable, there is more conservation in the core oligosaccharide region and, especially, in lipid A structure (Gronow and Brade, 2001). In lipid A, variations occur in the number and structure of acyl groups attached to the diglucosamine moiety and also in the numbers and degree of substitution of phosphate groups (Fig. 3). The core oligosaccharide can also vary with respect to phosphorylation and substituents attached to the conserved Kdo.

The importance of LPS structure in determining resistance to AMPs first became clear in studies of *Salmonella enterica* serovar Typhimurium, in which environmentally regulated two-component signal transduction pathways (*phoPQ*, which in turn regulates another two-component pathway, *pmrAB*) cause LPS modifications that decrease binding and killing by AMPs and increase pathogenicity (Ernst et al., 1999). These modifications include the partial charge neutralisation of lipid A by addition of 4-deoxy-4-aminoarabinose (Ara4N) to the phosphate residues attached to the diglucosamine, and addition of palmitate to lipid A results in alterations to membrane fluidity and self-promoted uptake of AMPs. Environmentally regulated modification of myristate in lipid A to hydroxymyristate is thought to be responsible for reduced host cell recognition by LPS (Ernst et al., 1999).

LPS structure also contributes to AMP resistance in a number of respiratory pathogens. *P. aeruginosa* possesses systems for environmental moderation of its LPS to more resistant phenotypes (Ernst et al., 1999; Macfarlane et al., 2000; Moskowitz et al., 1999). Lipid A from *P. aeruginosa* isolates from CF patients was highly substituted with Ara4N and was further altered through the addition of an acyl group (Ernst et al., 1999; Pier, 2000). The innate resistance of *B. cepacia* to AMPs may be in large part explained by the structure of its LPS. This organism has been shown to lack a self-promoted uptake pathway for AMPs (Hancock,

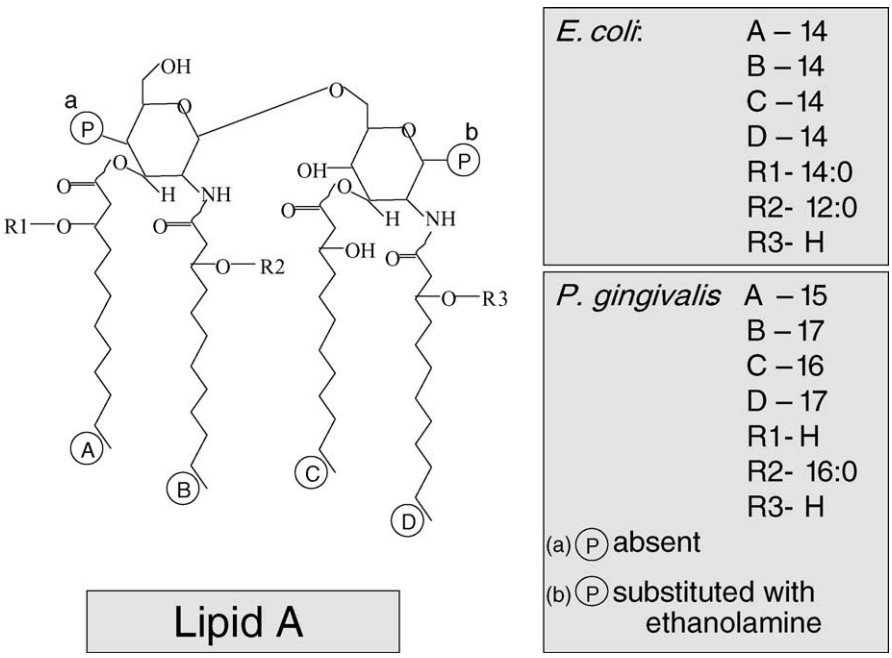

Fig. 3. Structure of *E. coli* and *P. gingivalis* lipid A.

1998) and its LPS binds AMPs poorly (Albrecht et al., 2002). *B. cepacia* LPS is relatively under-phosphorylated and the polysaccharide core, which consists of Kdo linked to D-glycero-α-D-talo-oct-2-ulopyranosonic acid (Ko) rather than Kdo–Kdo typical of enteric bacteria and *Pseudomonas* spp., is substituted with Ara4N (Gronow and Brade, 2001; Albrecht et al., 2002). The related and highly virulent *Burkholderia pseudomallei*, which causes severe pulmonary infection, is also resistant to AMPs and this has been linked to LPS structure (Burtnick and Woods, 1999). Extracellular *Legionella pneumophila* are resistant to polymyxin B, but sensitive mutants were defective in expression of a gene with homology to *S. enterica* serovar Typhimurium *pagP* (Robey et al., 2001), which encodes a palmitoyl transferase that is responsible for addition of an acyl group to lipid A. Unencapsulated non-typable *H. influenzae* (NTHi) is a common commensal of the upper respiratory tract and is sometimes associated with localised disease, and it has been shown to regulate acylation of lipid A. Under-acylated mutants of *H. influenzae* were more susceptible to killing by HBD2, but killing by the more highly cationic HBD3 was unaffected (Starner et al., 2002). Swords et al. (2002) found that such mutants were also less able to colonise human airway xenografts, and speculated that NTHi may differentially acylate lipid A during commensal and disease states.

The oral pathogen *P. gingivalis* does not induce expression of HBD2 by oral epithelial cells (Krisanaprakornkit et al., 1998) and appears to be quite resistant to some AMPs, such as human LL-37 and sheep SMAP-29 (Guthmiller et al., 2001). Although it was strongly inhibited by cecropin B, this activity was bacteriostatic rather than bactericidal (Devine et al., 1999). *P. gingivalis* possesses LPS that is unusual in many respects (Fig. 3). Its lipid A carries five acyl chains, which are unusually long and branched compared with those typical of enteric bacterial LPS, and the diglucosamine has only one phosphate group (Ernst et al., 1999; Netea et al., 2002). In addition to having the potential to contribute to AMP resistance, it has been proposed that these structural differences result in the different cellular responses initiated by *P. gingivalis* LPS compared with *Escherichia coli* LPS, and the recognition of *P. gingivalis* LPS by Toll-like receptor 2 (TLR2) rather than TLR4 (Ernst et al., 1999; Pulendran et al., 2001; Netea et al., 2002; Ogawa et al., 2002). Our preliminary results indicate that *P. gingivalis* LPS is also environmentally regulated and this regulation results in changes in the ability of LPS to induce cytokines from monocytes (Percival et al., 2001). We are currently investigating the structure of LPS from this organism using electrospray mass spectrometry in relation to environmental regulation and interactions with host cells and AMPs.

Studies of *E. coli* have shown that the O-polysaccharide chemotype significantly effects interactions, for example, with complement mediated innate defences (Devine and Roberts, 1994). Most studies of LPS and AMPs have pointed to the over-arching importance of properties of the lipid A and core oligosaccharide, but there are some instances in which the O-polysaccharide may be influential. Studies of salmonellae showed that reduced susceptibility to magainin was related to reduced O-polysaccharide chain length (Rana et al., 1991). AMP resistance of a respiratory pathogen, *Bordetella bronchiseptica*, was dependent on expression of genes involved in synthesis of the O-polysaccharide (Banemann et al., 1998). In *P. gingivalis*, this molecule may influence AMP interactions in a different way. This organism has a highly unusual O-polysaccharide, in which 60% of the α-rhamnose residues in the repeating unit are phosphorylated through addition of phosphoethanolamine (Paramonov et al., 2001). The consequent increased negative charge of the LPS may be expected to increase the binding of AMPs to the outer leaflet of the outer membrane; this may contribute to resistance by preventing or delaying access of AMPs to the core oligosaccharides and lipid A. Also, *P. gingivalis* expresses membrane-bound proteases and binding to the O-polysaccharide could influence the likelihood of inactivation of AMPs by such molecules. The relevance of proteases is discussed later.

4.2. Other cell wall properties

Many organisms that colonise the oral cavity and upper respiratory tract can decorate their cell surfaces with host-derived phosphorylcholine (ChoP). This form of host-mimicry is displayed by streptococci, pneumococci, *Neisseria* spp., *Haemophilus* spp., *Actinomyces* spp., *Fusobacterium* spp., mycoplasmas and others (Lysenko et al., 2000; Schenkein et al., 2001). ChoP substitution of *H. influenzae* LPS resulted in reduced sensitivity to LL-37 (Lysenko et al., 2000), but did not affect sensitivity to HBD2 or HBD3 (Starner et al., 2002). Both *H. influenzae* and *S. pneumoniae* variants expressing ChoP were more efficient than ChoP$^-$ variants at colonisation and persistence in animal models. ChoP was detected on the surfaces of a large number of commensal and pathogenic *Neisseria* spp. (Serino and Virji, 2000). However, in commensal strains ChoP was only present on LPS, whilst in pathogenic strains it decorated pili. In these strains, ChoP decoration was further important because cell surface ChoP facilitated adhesion to platelet activating factor on host cells and also affected the host signalling pathways initiated by bacterial colonisation (Swords et al., 2002).

Gram-positive cell wall teichoic acids and lipoteichoic acids are variably modified with alditol groups by glycosyl residues or D-alanine. Strains of *Staphylococcus* spp. were less sensitive to AMPs following *dlt* operon-mediated D-alanyl esterification of teichoic acids, which decreased cell wall negative charge and consequently reduced AMP binding (Peschel et al., 1999). A *dlt*-deficient mutant of *S. aureus* was less virulent in a mouse model than the wild type strain (Collins et al., 2002). Many Gram-positive species possess *dlt* operons and this may be a common mechanism for resisting innate peptides, as well as peptides produced

by other Gram-positive bacteria, such as lactococcin, nisin and subtilin (Sahl et al., 1995; Nes et al., 1996).

4.3. Protease production

Many oral and respiratory bacteria, and some important pathogens, are strongly proteolytic. Extracellular proteases contribute to nutrient acquisition, tissue destruction and deregulation of inflammatory responses (Potempa et al., 2000). Organisms with proteolytic metabolism also have efficient peptide uptake and transport mechanisms; thus, there is the potential for such organisms to be protected from antimicrobial peptides through direct proteolytic degradation or by uptake and transport systems binding and diverting antimicrobial peptides away from target sites.

A relationship has been observed between AMP sensitivity and extracellular protease production by some respiratory pathogens. Schmidtchen et al. (2002) demonstrated proteolytic degradation of LL-37 by a number of pathogens including *P. aeruginosa* and *Streptococcus pyogenes*. In *P. aeruginosa*, this was related to elastase production and degradation also correlated with sensitivity to killing by LL-37. Elastase was additionally significant in that it induced processing of the LL-37 neutrophil precursor proprotein, hCAP18, to active LL-37. Extracellular protease production by *B. cepacia* has been reported to contribute to antibiotic resistance (Hayashi et al., 2000). We found that eight strains of *B. cepacia* representing six genomovars were not strongly proteolytic and did not degrade β-defensin 1, LL-37, SMAP-29 or D2A-22 (unpublished results).

Oral subgingival anaerobes produce proteases that contribute to their nutritional requirements and to their abilities to subvert host defences. Strains of *P. gingivalis*, *Prevotella intermedia*, *Prevotella nigrescens*, *Prevotella corporis* and *Prevotella pallens* secreted proteases that cleaved and inactivated a number of AMPs while oral Gram-positive organisms, such as streptococci, actinomyces and *Rothia dentocariosa*, did not (Devine et al., 1999). Although *P. gingivalis* proteases are strongly down-regulated by environmental temperature, alterations in growth temperature still resulted in sufficient protease production to completely inactivate SMAP-29 and D2A-22 (Percival et al., 1999; unpublished results). The relationship between *P. gingivalis* protease production and sensitivity to AMPs is complex. The MIC of cecropin B did not correlate with an ability to degrade AMPs by extracellular proteases (Devine et al., 1999), although for each of the oral anaerobes tested the peptide was inhibitory but not bactericidal. It was suggested that this might have been partly because *P. gingivalis* proteases inactivated cecropin B slowly (10–15 min for full inactivation), whereas AMPs generally act rapidly. *Treponema pallidum* is a protease-producing oral spirochete associated with periodontal disease that lacks LPS. It was resistant to β-defensins, but this was not due to protection by extracellular proteases (Brissette and Lukehart, 2002). Thus, it appears that in some protease-producing oral bacteria, inner membrane characteristics and LPS structure are of over-riding importance in determining sensitivity to AMPs. Nonetheless, their proteases may provide indirect protection from AMPs and other host defences. Most Gram-negative bacteria release membrane-bound vesicles from their cell surfaces and those of *P. gingivalis*, *P. intermedia*, *P. nigrescens* and *P. aeruginosa* contain proteases (Devine et al., 1989; Mayrand and Grenier, 1989; Beveridge, 1999). Thus, proteases may be released and act at sites distant to surfaces of the producer cells, providing protection to accompanying species and deregulating host defences over a broader area. *P. aeruginosa* elastase and alkaline proteinase, *S. pyogenes* cysteine proteinase and *Enterococcus faecalis* gelatinase degrade host cell proteoglycans, releasing dermatan sulphate which inhibits HNP1 (Schmidtchen et al., 2002). *P. gingivalis* protease degrades CD14, thereby rendering host cells non-responsive to LPS (Tada et al., 2002) and preventing induction AMPs and other host defence molecules.

Most of the earlier studies have considered extracellular proteases, but outer membrane associated proteases were shown to protect *E. coli* and *S. enterica* serovar Typhimurium from the lethal actions of certain AMPs (Stumpe et al., 1998; Guina et al., 2000). *P. gingivalis* proteases are expressed extracellularly and are membrane bound (Curtis et al., 1999, 2001) and these proteases may contribute to protection. The strong negative charge of the phosphorylated O-polysaccharide may enhance AMP binding without increasing cell death because of protection by membrane-bound proteases.

5. Environmental regulation of bacterial properties relevant to interactions with antimicrobial peptides

Many of the cellular properties described earlier that contribute to AMP resistance are modified in response to alterations in environmental stimuli. Two-component signal transduction pathways that induce resistance to AMPs and are homologous to those responsible for environmentally regulated changes to *S. enterica* serovar Typhimurium LPS have been found in other enteric organisms as well as in *P. aeruginosa*, *Neisseria meningitidis*, *L. pneumophila* and *B. pseudomallei*. The modifications associated with AMP resistance may affect other host–microbe interactions that are also highly significant in determining the outcomes of microbial colonisation. Modifications to acyl moieties of lipid A strongly influence interactions between LPS and host receptors, thereby helping determine the signalling pathways initiated. It has been proposed that acylation affects the shape of lipid A, which in turn determines binding to host cell Toll-like receptors (Netea et al., 2002). Under-acylated mutants of NTHi stimulated host cells less than wild type bacteria and elicited a less pro-inflammatory response (Swords et al., 2002). ChoP on teichoic acids of *S. pneumoniae* and on LPS of NTHi facilitates adhesion to platelet activating factor on host cells, and the products of *dlt* provide other

selective advantages to some bacteria, such as increased acid tolerance, greater intracellular polymer accumulation, and mediation of interbacterial aggregations involved in biofilm establishment (Clemans et al., 1999; Spatafora et al., 1999; Boyd et al., 2000). Proteases can have many indirect effects beyond direct damage of host tissues and AMPs, including deregulation of inflammatory processes, and inactivation of host cell receptors. *P. gingivalis* proteases, and other virulence determinants, are environmentally controlled and appear to be down-regulated by conditions mimicking inflammation; this attenuation of virulence under certain conditions may contribute to the long-term survival of this organism within the hostile environment of the periodontal pocket (Percival et al., 1999; Bonass et al., 1999).

It is not really clear why some epithelial AMPs appear to be expressed constitutively while others require bacterial and inflammatory mediators for induction. HBD1 is much less potent as an antimicrobial agent than inducible HBD2 (van Wetering et al., 1999). It may be that other functions of HBD1, such as LPS binding, are more important. HBD1 may function as a constitutive "sentinel" AMP, binding to LPS and LTA, neutralising them, thereby helping prevent an undesirable immune response to low levels of organisms or to resident commensal bacteria. Most bacteria colonising tissues contain LPS or LTA in their cell walls. It is becoming clear that subtle differences in LPS structure, many of which are environmentally regulated, have significant impacts on interactions with host cells and synthesis of host molecules, including inducible AMPs. It remains to be seen how these structural properties influence binding to AMPs and the subsequent interactions between AMP–LPS complexes and host cells, such as dendritic cells, and how this impacts on consequent host responses.

Thus, oral and respiratory AMPs interact with large numbers of colonising bacteria. The diversity of these populations can be immense and, in addition to the species diversity evident in resident populations, single species exhibit substantial genetic diversity (e.g. Jolley et al., 2000; Hohwy et al., 2001). The complexity of host–microbe interactions is further increased by the fact that micro-organisms rapidly adapt to changing environmental conditions, in ways that may increase survival and pathogenicity. Given the complexities of these microbial populations and their genetic flexibility, it is essential that host defences are equally varied and flexible. AMPs fulfil these requirements and evidence is accumulating that demonstrates their importance in protecting against specific pathogens, modulating resident populations and in regulating host responses to bacteria and their products, especially LPS.

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