Human Beta-Defensin-3: A Promising Antimicrobial Peptide

G. Batoni*, G. Maisetta, S. Esin and M. Campa

Dipartimento di Patologia sperimentale, Biotecnologie mediche, Infettivologia ed Epidemiologia, Via S. Zeno 35-39, University of Pisa, 56127 Pisa, Italy

Abstract: The field of naturally occurring antimicrobial peptides is a research area rapidly expanding due to the high potential of such molecules as new antimicrobial drugs. In this regard, the human beta-defensin-3 is particularly attractive because of its strong antibacterial activity, relative salt-insensitiveness and low toxicity for host cells.

Key Words: Antimicrobial peptides, human beta-defensin-3, drug resistant microorganisms, new antimicrobial drugs, peptide structure.

INTRODUCTION

Naturally occurring cationic antimicrobial peptides (AP) are essential components of the innate immune system and are found at all levels of the evolutionary scale [1]. Some important characteristics of such peptides have suggested their use as a new class of antimicrobial drugs. Among these, there are the broad activity spectrum, the relative stability, the selectivity towards microbial targets, the rapid mechanism of action, the low frequency in selecting resistant strains, and the ability to have a synergistic activity with conventional antibiotics [2]. The need of identifying new molecules with antimicrobial activity is largely justified by the growing appearance and rapid spread of pathogenic microorganisms resistant to available drugs that represent, at present, a serious public health problem worldwide [3].

Among human AP, defensins are the family including the highest number of peptides. They have a molecular weight of 3.5-6 kDa and contain six cysteine residues that form three intramolecular disulfide bridges [4, 5]. Two main subfamilies of defensins exist, the α- and β-defensins, which differ in the location and position of the cysteine residues, in the amino acid sequence, and in their difulfide motifs [5]. The α-defensins are most commonly found in neutrophils (human neutrophil peptide, HNP1-4) and in Paneth cells of the small intestine (human defensins 5 and 6) [4]. In contrast, human β-defensins are expressed predominantly by the epithelial cells of many organs, including glandular and squamous epithelia [4]. Because of their preferential localization at epithelial surfaces, β-defensins are believed to play an essential role in the defense of the mucosal barrier from infections. The first human β-defensins (HBD1 and HBD2) were discovered in 1995 and 1997, respectively [6, 7]. HBD1 was isolated from human hemofiltrate of patients undergoing hemodialysis for renal failure [6] and was found to be constitutively expressed by epithelial cells of many organs [8, 9]. HBD2 was originally purified from psoriatic skin lesions, a source particularly rich in human skin-derived antimicrobial proteins [10], based to its binding to a whole *Escherichia coli* affinity column [7]. In contrast to *HBD1*, *HBD2* gene was found to be upregulated following microbial or inflammatory stimuli [7]. In 2001 three distinct research groups independently identified HBD3 by using different approaches [11, 12, 13]. In an attempt to screen human epithelia for endogenous factors with *Staphylococcus aureus*-killing activity, Harder and collaborators isolated the peptide from psoriatic scales, by biochemical methods [11]. By passing crude lesional scale extracts of patients with psoriasis through a *S. aureus* affinity column and purification of the staphylocidal activity bound to the column, they were able to purify 88 μg of HBD3 from 7 g of psoriatic scales. Independently, Garcia *et al*. [12] and Jia *et al*. [13] detected HBD3 by screening human genomic sequences for a gene with homology to the *HBD1* and *HBD2* genes. In 2001 the fourth human β-defensin (HBD4) was also described using bioinformatic and functional genomic analysis [14]. Analysis of the distribution of HBD4 transcripts in the human body revealed a restricted pattern of expression with the highest levels of transcripts found in the testis and in the gastric antrum. One year later, screening of human genomic sequences for the presence of structural motifs of defensins allowed for the identification of two further human β-defensins (HBD5 and HBD6) specifically expressed in the epididymis [15]. By using a computational search strategy, Schutte *et al*. [16] could identify 28 new human β-defensin genes allocated in five conserved gene clusters. Thus, much work has yet to be done to characterize the entire array of β-defensin gene products and functions in humans.

Extensive overviews of human defensins have been published over the last few years [4, 5, 17]. This review will focus on the recently described HBD3. Since its discovery, a bulk of information has accumulated about HBD3 structural properties, biological activities, gene expression, role in innate and adaptive immunity. Special emphasis will be placed on the distinctive properties of the peptide that make it particularly attractive as a potential antimicrobial agent.

STRUCTURE OF HBD3

HBD3 is a peptide of 45 amino-acidic residues with a molecular mass of 5.15 kDa containing, as the other β-defensins, a conserved six-Cys motif in which the six cys-

*Address correspondence to this author at the Dipartimento di Patologia sperimentale, Biotecnologie mediche, Infettivologia ed Epidemiologia, *Via* S. Zeno 35-39, University of Pisa, 56127 Pisa, Italy; Tel: +39 0502213694; Fax: +39 0502213711; E-mail: batoni@biomed.unipi.it

1389-5575/06 $50.00+.00

teines are linked at the 1-6, 2-4 and 3-5 locations to form three disulfide-bridges [18]. HBD3 has an exceptionally high net positive charge (+11) due to the presence of 7 Arg and 6 Lys residues (Fig. **1**). The peptide has an amphiphilic structure in which the positive charges are asymmetrically positioned on the two sides of its surface, while the hydrophobic residues are clustered at the bottom of the peptide surfaces [18]. The secondary structure of HBD3 is characterized by the presence of six disordered N-terminus residues, followed by a short helical segment, composed of residues positioned at 10-14 and by three-stranded anti-parallel β-sheets formed by residues 17-19, 27-31, 39-43, respectively (Fig. **1**) [18]. Spectroscopic analyses have shown that the disulfide connectivity has no significant effect on the secondary structure of HBD3, as demonstrated by the absence of structural changes even when Cys residues are substituted by other amino-acidic residues [18]; thus, the secondary structure of HBD3 seems to be preformed independently by the presence of disulfide bridges [18]. The packing of the helical segment against the β-sheet is primarily constrained by the disulfide bond between Cys 11 and Cys 40. Nuclear magnetic resonance data suggest that HBD3 in solution forms a symmetrical dimer through strand β2 of the β-sheet [18]. Such a property is confirmed by electrophoretic separation in tricine gels where, before reduction of the disulfide bonds, the mobility of HBD3 corresponds to that of a dimer (10 kDa), whereas after reduction of the disulfide bonds with dithiothreitol the mobility of HBD3 corresponds at its real molecular weight (5 kDa) [17, 18]. This observation indicates that cleavage of the disulfide bonds results in a disruption of the tertiary structure of HBD3 and the subsequent loss of stable dimers.

It is generally agreed that the antimicrobial activity of β-defensins relys on their ability to bind the bacterial surface by electrostatic interactions and to insert themselves into the membrane by hydrophobic interactions [17]. In an attempt to study the correlation between antimicrobial activity and structural properties of HBD3, Klüver *et al*. [19] tested the antibacterial activity of a number of synthetic analogues of HBD3 differing in length, charge, disulfide connectivity and overall hydrophobicity. Interestingly, they observed that peptides with similar secondary structures exhibited significant variety in their antimicrobial potency, suggesting that the antimicrobial effects of HBD3 are due to the composition of the peptide rather than to its spatial arrangement.

HBD3 TISSUE DISTRIBUTION

HBD3 seems to be less widely expressed than other human β-defensins such as HBD1 and HBD2. Harder and collaborators investigated the tissue distribution of HBD3 mRNA expression from various body sites by real-time RT-PCR and found low or no HBD3 mRNA expression in most of the analyzed organs including the respiratory, gastrointestinal, and genitourinary tracts [11]. In contrast, the same Authors detected strong expression of the peptide in skin and tonsils. Despite the low HBD3 expression in biopsies from gastrointestinal tract [11], purified epithelial cells of normal small and large intestine were found to express high level of HBD3 mRNA [20]. HBD3 mRNA is also expressed in corneal epithelial cells [21] and in human endometrial epithelium, predominantly during the secretory phase of the menstrual cycle [22]. Analysis of HBD3 mRNA expression has also been perfomed in oral tissues where the peptide has been found to be widely expressed [23]. In particular, HBD3 transcripts were detected in the majority of non-inflamed oral tissues analyzed, including gingiva, tongue, buccal and labial mucosa, and dental follicles, but not in dental pulp and only at low frequency in salivary glands [23]. Studies in primary cell cultures demonstrated that oral keratinocytes, but not fibroblasts, contain transcripts for HBD3 suggesting that, similarly to what was observed for HBD1 and -2, expression of HBD3 is probably restricted to the epithelial compartment of the oral cavity. Gene expression levels of HBD1, -2 and 3 mRNAs vary considerably from one individual to another, but correlate with each other in the gingival tissues of young children [24]. The wide expression of HBD3 in oral tissues, together with its coordinated expression with other defensins at this level, suggest that this peptide might be part of the oral host defense which contributes

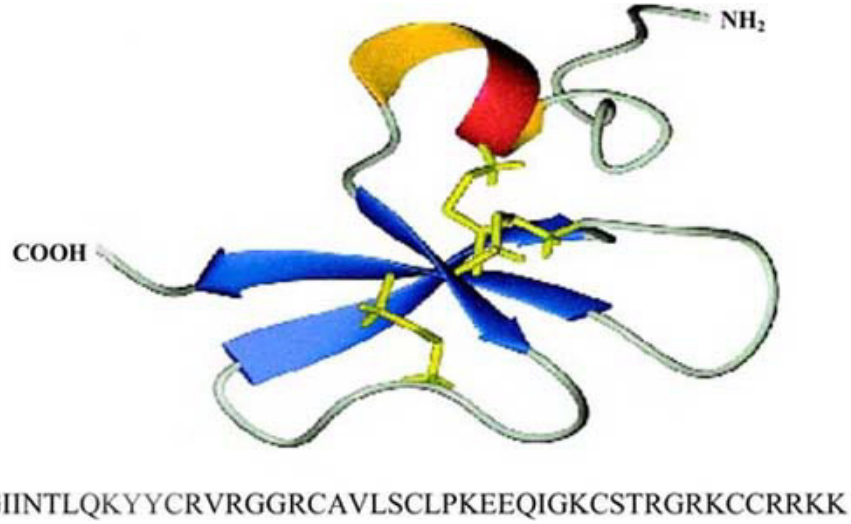

Fig. (1). Ribbon diagram and aminoacidic sequence of HBD3; the three disulfide-bonds are shown in gold. Reproduced from Schibli *et al*. [18] with permission.

to the ecologic balance within the oral cavity, a site regularly colonized by different microorganisms with pathogenic potential.

In addition to epithelial tissues, Garcia *et al*. [12] demonstrated expression of HBD3 in some non-epithelial tissues, such as neutrophils, heart and skeletal muscle. Despite the basal expression described by Garcia *et al.* in neutrophils, HBD3 gene was not found to be either expressed or induced in whole human peripheral blood by Fang *et al*. [25].

Although several studies have analyzed HBD3 gene expression at the mRNA level, only few reports investigated the expression of the peptide at the protein level. Harder *et al*. were able to purify about 10 μg of HBD3 from the supernatants of 10^9 cultured primary keratinocytes as well as from 10^9 A549 lung epithelial cells previously pretreated with *Pseudomonas aeruginosa*, indicating that skin keratinocytes and cells of respiratory tract represent cellular source for HBD3 *in vivo* [11]. Very recently, HBD3 was demonstrated to be present in unstimulated saliva of children by immunoblot technique using a polyclonal serum [26]. Although considerable variation was observed among different individuals (median 0.31 μg/ml, range from 0 to 6.21 μg/ml), the detected levels of the peptide are in the range of effective antimicrobial function, further suggesting a role for HBD3, alone or in combination with other salivary AP, in the maintenance of the oral cavity health.

MODULATION OF HBD3 EXPRESSION

Usually, β-defensin gene expression is considered either constitutive or inducible. As opposed to HBD1, which was found to be expressed constitutively in many epithelial tissues, and similarly to HBD2, the mRNA expression of HBD3 is regulated by a variety of microbial and host factors [11]. The contact of keratinocytes or primary tracheal epithelial cells with heat inactivated Gram-negative or Gram-positive bacteria like *P. aeruginosa* and *Staphylococccus aureus*, respectively, induced HBD3 mRNA [11]. Modulation of β-defensin mRNA was also investigated following stimulation of primary and/or immortalized oral epithelial cells with periodontal pathogens such as *Actinobacillus actinomycetemcomitans* [27] and *Porphyromonas gingivalis* [28]. Both bacterial species caused an increase in *HBD3*, but not *HBD2* gene expression suggesting that HBD2 and HBD3 may have distinct pathways of activation. Ability to up-regulate *HBD3* gene and/or peptide expression was also described for *Campylobacter jejuni*, the most prevalent cause of bacterial diarrhoea worldwide [29], for rhinovirus-16, a respiratory virus responsible for the common cold and associated with asthma exacerbations [30], and for HIV-1 [31].

In addition to microbial stimuli, β-defensin expression may be modulated by inflammatory stimuli or other host factors [4, 5]. In contrast to HBD2, which is mainly up-regulated by interleukin 1 beta (IL1-β) and tumor necrosis factor alfa (TNF-α), HBD3 expression increases particularly after stimulation with interferon gamma (IFN-γ) [12, 32]. Combination of IFN-γ with either IL1-β or TNF-α results in synergistic induction of HBD3 mRNA expression in primary gingival keratinocytes cultures [32]. In *in vitro* cultures of primary endometrial epithelial cells, HBD3 mRNA expression is upregulated by treatment with inflammatory molecules including IL1-β plus TNF-α, IFN-γ, and phorbol ester suggesting that, together with other AP found at this level, HBD3 may participate to the mucosal defense system present in the uterus [22]. Other host factors which were found to induce *HBD3* expression include IL-22, a cytokine produced mainly by activated T and natural killer (NK) cells [33], and transforming growth factor alfa (TGF-α), alone or combined with insulin-like growth factor I (IGF-I), two important growth factors involved in wound healing [34].

Joly *et al*. [32] recently demonstrated that in addition to a variable basal expression of β-defensin mRNA among individuals, the mRNA induction potential of a given β-defensin may also vary between one subject and an other, but correlates with its basal expression. This observation suggests that health at mucosal surfaces is best maintained in individuals presenting high basal expression levels of the defensins as these are the individuals who will also have the potential to produce higher amount of such peptides in the presence of inflammatory mediators. Some studies on the analysis of HBD3 expression in pathologic conditions as compared to healthy tissues seem to support this hypothesis. For instance, Bissell *et al*. [35] compared mRNA levels in gingival health and in periodontal disease and found significantly higher levels of HBD3 expression in the healthy tissues compared to the diseased ones. This finding is consistent with a previous study in which HBD3, as well as other human β-defensins, were found to be expressed at higher frequency in non-inflamed oral tissues than in inflamed tissues [23]. In Crohn's disease, a deficiency in HBD2 and HBD3 induction has been hypothesized to contribute to an impaired mucosal bacterial barrier [36, 37].

Several factors have been proposed to be responsible for the variable expression/induction patterns of the defensins observed among different individuals in health and disease. These include differential responses to inducers, cytokine or defensin down regulation during infections as part of microbial evasion mechanisms of the host defense [38], host protease-mediated destruction of defensins [39], genetic polymorphism [40] or variable defensin gene copy numbers [41]. The considerable variation among induction levels as well as the correlation of induction with basal expression [32] suggest that β-defensins may represent innate response elements which play a key role in susceptibility or resistance to diseases at the epithelial surfaces. If this will be proved to be true, immunoprophylactic strategies based on exogenous administration of β-defensins in subjects genetically determined to produce low levels of these peptides, might be considered in the future.

The signaling pathways involved in regulation of HBD3 expression are not yet well understood. The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, the main signaling mechanism for a wide array of cytokines and growth factors [42], has been demonstrated to be involved in IFN-γ- mediated up-regulation of HBD3 expression [32]. This finding is compatible with the presence of a STAT binding site at the 5' genomic sequence of the *HBD3* gene [12] and with the inhibition of *HBD3* gene expression by corticosteroids [43] which are known to inhibit the cytokine signaling *via* the JAK/STAT pathways [44].

In conclusion, the *in vivo* β-defensin response to infection/inflammation is likely to be modulated by multiple and complex regulatory pathways which are at the moment only partially defined.

BIOLOGICAL FUNCTIONS

(i). Antimicrobial Activity

A number of *in vitro* studies has demonstrated a marked antibacterial, antifungal and antiviral activity of HBD3 [45, 46, 47, 48]. Unlike HBD1 and HBD2, the microbicidal activity of which is directed predominantly against Gram-negative bacteria, HBD3 exhibits a broad range of antibacterial activity against both Gram-positive and Gram-negative bacteria at concentrations much lower than those observed for other members of the β-defensin family. Table **1** depicts the minimum bactericidal concentrations (MBCs) of HBD3 against several clinically relevant bacterial strains at date tested. Although different methods can be used to assess the ability of peptides to kill bacteria, most of the MBC values reported in the table have been obtained using a conventional microdilution method in sodium phosphate buffer (pH=7.4) [47]. For Gram-negative bacteria, the MBCs range from 1.56 to 16 μg/ml, while for Gram-positive the MBCs range from 0.4 μg/ml to 6.25 μg/ml. HBD3 antibacterial spectrum of action also includes oral Gram-positive and Gram-negative bacteria involved in caries or periodontal diseases such as *Streptococcus sobrinus*, *Lactobacillus acidophilus*, *Streptococcus mutans*, *A. actinomycetemcomitans* [46, 47, 49]. Among oral bacteria, *S. mutans* is the most susceptible to HBD3, while *P. gingivalis* is reported to be quite resistant in different studies [46, 47, 49]. By using a radial diffusion assay, Joly *et al.* evaluated the minimum inhibitory concentrations (MICs) of HBD3 against a wide collection of oral organisms [46]. Interestingly, by testing at least three strains within each species they demonstrated a strain-specific rather than species-specific activity of HBD3 (and HBD2) against Gram-negative bacteria associated to periodontal diseases (*P. gingivalis*, *A. actinomycetemcomitans*), a finding only partially confirmed by others [49]. The modulation of surface molecules (i.e. degree of lipid A acylation) even within one species has been reported to correlate with susceptibility to AP (50). Slight differences in the composition of surface structures among strains belonging to the same species may help to explain the observed strain-specific susceptibility to HBD3 of oral bacteria. It might be argued that, due to the wide expression of β-defensins in the oral cavity [24, 35, 51], oral bacteria have evolved a certain extent of structural variability which allow them to evade the local antimicrobial host immune response.

Recently, we have tested the bactericidal activity of HBD3 against several clinical isolates of nosocomial pathogens resistant to most classes of antibiotics (multi drug-resistant, MDR) (Table **1**) [48]. Interestingly, HBD3 demonstrated bactericidal activity towards all clinical isolates tested (6 strains for every species) at concentrations between 4 and 8 μg/ml, irrespective of the resistance profile exhibited. In contrast to the strain-to-strain variability of HBD3 activity observed by Joly *et al*. against oral bacteria [46], minimal degree of variation in MBC values of HBD3 was observed among different strains belonging to the same species [48].

As reported in Table **1**, among the bacterial strains tested so far, *Serratia marcescens* exhibits an intermediate susceptibility to HBD3 (MBC 50 μg/ml), while *Burkolderia cepacia* is resistant to the peptide (MBC>100 μg/ml). *Burkholderia* spp. are known to resist cathelicidins [52], HBD1 and HBD2 and other cationic peptides [53]; the resistance of *Burkolderia* spp. to cationic peptides has been suggested to be in part due to structural peculiarities of the lipopolysaccharide (LPS) of such bacteria. In particular, cationic substitutions of LPS that produce a reduction in the net negative charge of the external membrane of the bacterium could impair the affinity of the bacterial surface for positively charged cationic peptides [45].

Owing to their mechanism of action, antimicrobial cationic peptides exhibit, in general, faster killing kinetics than conventional antibiotics [2]. In order to assess whether this was also the case for HBD3, we investigated the killing kinetics of the peptide in sodium phosphate buffer against a number of Gram-positive and Gram-negative species [48]. Interestingly, HBD3 showed a very fast bactericidal activity against both groups of bacteria, although with slightly different kinetics. Infact, the peptide was bactericidal within 1 to 5 min. against Gram-negative species (*Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii*) and within 10 to 20 min. against Gram-positive species (*S. aureus* and *Enterococcus faecium*) indicating a slower bactericidal mechanism of HBD3 against Gram-positive than Gram-negative bacteria. This difference in killing kinetics may be due to structural differences between the two groups of bacteria, and may indicate that the outer membrane and the thin periplasmic peptidoglycan layer of Gram-negative bacteria interfere less with HBD3 activity than the thicker peptidoglycan layer of Gram-positive microorganisms.

A number of reports indicate that host proteins with antimicrobial activity are able to work in concert *in vivo* to enhance their effectiveness in the protection of epitelia and mucosal surfaces by infections (47, 54, 55). Thus, evaluating the antimicrobial activity of a single peptide most likely underestimate its potential as an antimicrobial agent *in vivo* where synergistic interactions of diverse AP take place. HBD3 has been reported to exhibit *in vitro* a synergistic effect in combination with LL-37, an AP produced by neutrophils, and an additive effect in combination with HBD1 and HBD2 against *S. aureus* [56]. Furthermore, the bactericidal activity of HBD3 is enhanced by the presence of lysozyme when tested against *S. aureus* and *S. mutans* [47, 55]. A synergistic effect of HBD3 has been described also with commonly used antibiotics. Combination of suboptimal doses of β-lactam antibiotics, such as methicillin, with HBD3 resulted in a synergistic effect against methicillin sensitive and resistant *S. aureus* (MSSA, MRSA) [56]. Moreover, HBD3 has been tested in combination with metronidazole and amoxicillin, two antibiotics largely used in the therapy of periodontal diseases, or in association with chlorexidine, an oral disinfectant [47]. The ability of the peptide to enhance the bactericidal effect of all three antimicrobial agents was demonstrated against both periodontal pathogens (*A. actinomycetemcomitans* and *P. gingivalis*) and cariogenic species (*S. mutans*) [47]. Altogether these findings demonstrate an

Table 1. Susceptibility of Clinically Relevant Microorganisms to HBD3

Antimicrobial spectrum	Peptide MBC (μg/ml)	References
Gram-positive		
Staphylococcus aureus	1.56-8	[45, 48]
Staphylococcus epidermidis	1.56	[45]
Streptococcus pneumoniae	6.25	[45]
Streptococcus pyogenes	6.25-12	[45]
Enterococcus faecium	8-10	[48, 11]
Streptococcus mutans	2	[47]
Streptococcus sanguis	16	[47]
Streptococcus sobrinus	16	[47]
Lactobacillus acidophylus	8	[47]
Actinomyces naeslundii	4-7*	[46]
Actinomyces israelii	10*	[46]
Gram-negative		
Escherichia coli	0.4	[45]
Pseudomonas aeruginosa	1.56-8	[45, 48]
Pseudomonas putida	6.25	[45]
Stenotrophomonas maltophilia	0.78-8	[45, 48]
Acinetobacter baumannii	0.78-4	[45, 48]
Hemophylus influenzae	7.4*	[60]
Burkolderia cepacia	>100	[45]
Klebsiella pneumoniae	0.1	[45]
Serratia marcescens	50	[45]
Citrobacter freundii	0.39	[45]
Actinobacillus actinomycetemcomitans	2.5	[47]
Porphyromonas gingivalis	5.7->250*	[49, 47, 46]
Porphyromonas micros	15.9->250*	[49, 46]
Fusobacterium nucleatum	4.5->250*	[49, 46]
Fungi		
Candida albicans	2.8-7.1*	[46]
Candida parapsilosis	1.4-12.4*	[46]
Candida glabrata	33.8->250*	[46]
Candida tropicalis	3.3-14.4*	[46]
Candida krusei	2-13.7*	[46]

* the values indicate MIC (minimum inhibitory concentration).

enhanced bactericidal effect of HBD3 when combined with several antibacterial compounds exerting different mechanisms of action. It can be argued that drugs causing a breakdown of the bacterial cell wall (i.e. amoxicillin, methicillin, lysozyme) might facilitate the access of HBD3 into the cytoplasmic membrane. Such a hypothesis is consistent with the observation that after exposure to suboptimal concentrations of methicillin (1/16 MIC), *S. aureus* cells are more sensitive to HBD3 than non-treated bacteria [56]. On the other hand, increased permeability of the outer and/or inner membranes caused by the peptide could facilitate the entry of antibiotics with an intracytoplasmic target (i.e. metronidazole, chlorexidine).

Antifungal activity of HBD3 has been described against a number of *Candida* spp. [46] (Table **1**), although the action mechanisms and fungal targets of HBD3 are unknown. The antifungal activity of HBD3 was found to be variable among strains of the same species and ranged from 1.4 μg/ml to > 250 μg/ml.

The exact mechanism(s) by which defensins exert their microbicidal activity has yet to be completely clarified. It is generally agreed that in the early phases of peptide-bacterium interaction basic aminoacids of the peptides bind to negatively charged molecules exposed on bacterial surfaces such as teichoic acids of Gram-positive bacteria or lipopolysaccharides of Gram-negative bacteria [57]. Following such electrostatic binding, peptides reach the bacterial cell membrane which is their principal target of action. The net negative charge exhibited by this structure, due to the presence of phosphatidylethanolamine and phosphatidylglycerol, and the high transmembrane potential (-140 mV) aid the interaction between bacterial membrane and AP. The selectivity of AP for bacterial membranes seems to rely on the high content of zwitterionic lipids (phosphatidylcholine and sphingomyelin) of eukaryotic membranes and consequently to a lower number of negative charges exhibited by the latter as compared to bacterial membranes [58]. Unlike amphipatic α-helical AP that cover the membrane in a carpet-like manner and dissolve it like a detergent, circumstantial evidence suggest that defensins interact with bacterial membranes by forming pores [58, 59]. This ability might derive from the formation of multimers by monomeric molecules of defensin (HBD3 form naturally dimers), which are electrophoretically driven into the membrane by the large electrical potential [58]. These channels increase the membrane permeability with loss of cytoplasmic content and cell death. Despite similarities in the general mechanism of antibacterial action of different β-defensins, experimental data suggest that possible differences among the β-defensins may also exist. For instance, mutant strains of *Haemophilus influenzae* for lipid A acylation (*htrB*) exhibit a sensitivity to HBD2 greater than 45-fold that of the wild type, while they exhibit similar sensitivity to that of the parental strain to HBD3 [60].

The crucial role of the negative charges on bacterial cytoplasmic membrane for the antimicrobial activity of human defensins, including HBD3, has been demonstrated by the analysis of *S. aureus* strains mutant for the *mprF*, and *lysC* genes. These strains have a reduced content of lysyl-phosphatidylglycerol in cell membrane and consequently a more negative net charge, and exhibit an increased susceptibility to HBD3 and/or other defensins [61, 62]. Similarly, a greater susceptibility to human defensins was observed in *S. aureus* mutant strains lacking D-alanine in their teichoic acids, as a result of which the cells carried an increased negative surface charge [63]. Moreover, recent work has shown that strains of *Staphylococcus epidermidis* mutant for the production of positively charged extracellular polysaccharides are significantly more susceptible to HBD3 than wild-type strains, further supporting the crucial role of negative surface charges for the antimicrobial activity of the peptide [64, 65].

Studies on membrane permeabilization by HBD3 have been carried out by following the kinetics of hydrolysis of extracellular chromogenic substrate by cytoplasmic β-galactosidase constitutively produced by recombinant strains of *E. coli* and *S. aureus* [66]. The results obtained have suggested that HBD3 is able to cause poration in membrane, and that the permeabilization of the cytoplasmic membrane is slower in Gram-positive than in Gram-negative microorganisms. These findings are in agreement with the killing kinetics of HBD3 that, as reported above, are slower in Gram-positive than in Gram-negative bacteria [48]. By electronic microscopy analysis, Harder *et al*. has revealed structural damages of cytoplasmic membranes of HBD3-treated *S. aureus* [11] further suggesting that bacterial membrane is one of the main targets for HBD3 antimicrobial activity. In conclusion, the enhanced antimicrobial potency of HBD3 is primarily due to the high net positive charge that facilitates the interaction between the peptide and the negative charged bacterial surfaces, and, secondly, to the increased capacity to form dimers in solution, compared with HBD1 and HBD2, which should enable HBD3 to cause an efficient disruption of bacterial membranes.

In an attempt to minimize the region necessary for antimicrobial activity of HBD3 for therapeutic use, several synthetic analogues of HBD3 of various sizes and with different disulfide pairing and charges have been tested against Gram-positive and Gram-negative species and fungi [19, 67, 68]. The antimicrobial activity of HBD3 resulted independent from its pattern of disulfide pairing and by the presence of cysteil residues [19, 68]. Moreover, the N-terminal extension of five aminoacids was not found to influence the antimicrobial activity of HBD3 peptides, while peptides corresponding to the C-terminus, the most basic, showed higher activities [19, 67]. Kluver *et al*. have proposed a classification scheme based upon the hydrophobicity-charge correlation of a number of HBD3 derivatives, into three groups: (i) peptides with a low net charge and moderate hydrophobicity are antimicrobially inactive; (ii) positively charged peptides with high hydrophobicity are potent antimicrobial agents although exhibiting cytotoxic effects on eukaryotic cells; (iii) HBD3 derivatives with high net charge and low hydrophobicity are strong AP causing no significant cytotoxic effects [19].

In addition to their microbicidal activity against bacteria and fungi, defensins exhibit also antiviral properties against certain enveloped viruses. HBD3 showed concentration-dependent inhibition of HIV-1 replication without cellular toxicity [31]. The inhibitory mechanism was investigated in details by Quinones-Mateus *et al*. [31] who demonstrated

that HBD3 binds directly to virions inducing irreversible inhibition of HIV replication and also binds to host cells inducing internalization of the CXCR4 chemokine HIV coreceptor. Inhibitory effect of HBD3 on HIV infectivity was also described by Sun *et al*. [69]. In this case, however, a decrease in cell proliferation was observed when high doses of HBD3 were used, leading to the possibility that the antiviral effect of HBD3 can be partially due to an influence on target-cell proliferation. The elucidation of the HIV-inhibitory activity of β-defensins and of their pattern of expression in the oral mucosa of HIV-negative and HIV-positive subjects will clarify whether such peptides may also contribute to protect oral mucosa by infection with such a virus.

(ii). Proinflammatory Activity

In addition to their antimicrobial effects, defensins have been shown to modulate a variety of cellular activities including chemotaxis of T cells, dendritic cells [70] and monocytes [12, 71], stimulation of epithelial cells and fibroblast proliferation [72, 73], stimulation of cytokine production [74, 75, 76], and release of histamine from mast cells [77]. These effects, which typically occur at defensin concentrations much lower than those required for antimicrobial activity, suggest that defensins may not only participate in the innate immune response system by virtue of their ability to kill microbes, but also as regulatory factors. HBD3 was tested as a potential chemoattractant for monocytes and neutrophils [12] and was found to induce migration of monocytes at nanomolar concentrations while it was not active on neutrophils. Chemotactic activity of human defensins relies on their ability to attract host cells expressing the appropriate receptors along a gradient to their site of origin. It has been demonstrated that, due to their chemokine-like structure, β-defensins may directly bind to and activate the chemokine receptor CCR6 preferentially expressed by immature dentritic cells and memory T cells and that this interaction results in chemotaxis of these cells [70]. HBD3 attracts human embryonic kidney cell lines (HEK 293) stably transfected to express CCR6 [68], suggesting that this molecule is one of the peptide receptors. As monocytes do not express CCR6, HBD3 must use at least one additional unidentified receptor besides CCR6 to attract such cells [78].

By virtue of their ability to recruit cells of both innate and adaptive immunity, defensins may act as important mediators of the *in vivo* host defense against infection. Nevertheless, when considering the use of defensins as antimicrobial agents, concern exists with regard to the potential side effects associated with their proinflammatory chemotactic properties which may follow exogenous administration of therapeutic doses of such peptides. In this regards it is noteworthy that Wu and collaborators [68] could dissect antimicrobial and chemotactic activities of HBD3 by engineering disulfide bridges of the molecule. They chemically synthesized six topological analogs of HBD3 with predefined disulfide connectivities. Unexpectedly, all differently folded HBD3 species exhibited similar antimicrobial activity against *E. coli*, whereas a wide range of chemotactic activities was observed with these analogs for monocytes and cells transfected by the chemokine receptor CCR6 [68]. Furthermore, whereas substituition of all cysteine residues by α-aminobutyric acid completely abolished the chemotactic activity of HBD3, the bactericidal activity remained unaffected in the absence of any disulfide bridge. Interestingly, the linear form of HBD3 was even more salt-resistant than the folded form [68], suggesting that abolishment of proinflammatory properties of HBD3 may probably be achieved without effecting those distinctive characteristics of the peptide (i.e. strong antibacterial activity and ability to retain it at physiologic salt-concentrations) which make it particularly promising as a new therapeutic agent.

MEDICAL AND CLINICAL APPLICATIONS

Due to the alarming spread of (multi)drug-resistant microorganisms worldwide, identification of new antibiotics is urgently needed. In this context, the possible use of natural AP as new class of antibiotics is taken into increasing consideration [79, 80, 81]. The therapeutic use of several AP is currently under investigation in animal models, while few peptides are being tested in clinical trials as topical or systemic antinfective agents [80]. For instance, P-113, a derivative of histatin, a human salivary peptide, is undergoing a phase II trial to test its ability to prevent gingivitis by mouthrinse formulation [82]. A protegrin-1 derivative is in phase II/III clinical testing for prevention of ventilator associated pneumonia [79]. Systemic administration of a cationic protein, produced by human polymorphonuclear leukocytes (bactericidal/permeability-increasing protein, BPI), has been tested in a randomized trial as adjunctive treatment for children with severe meningococcal sepsis due to its antimicrobial and endotoxin neutralizing activities [83]. Other possible applications of AP currently under investigation include skin treatment for prevention of catheter-related bloodstream infections, therapy of acute acnes, killing of MRSA in the nares or treatment of severe sepsis [79, 81, 84].

In this paragraph the distinctive characteristics of HBD3 which rationally justify the design of HBD3-based antimicrobial strategies will be summarized (Table **2**) and discussed. First of all, due to peculiar structural properties such as high positive surface charge and tendency to form dimers in solution, HBD3 exhibits a stronger and broader spectrum of antimicrobial activity than many other AP described so far. Such activity is directed against Gram-positive and Gram-negative bacteria, including multi-drug resistant isolates involved in nosocomial infections, as well as fungi and viruses. The ability to synergize with conventional drugs could be an advantage especially in the treatment of infections caused by seriously resistant pathogens. Based on *in vitro* experimental data [47, 55, 56] one could expect that a combination of HBD3 with partially active antibiotics *in vivo* could lower the bactericidal concentrations of the latter or even render susceptible antibiotic-resistant strains.

The antibacterial activity of β-defensins is normally sensitive to high (physiologic) salt concentrations. Unlike the other members of the β-defensin family, HBD3 is relatively salt-insensitive, i.e. maintains its antimicrobial activity at physiologic concentrations of NaCl [11, 17]. In particular, antimicrobial activity of HBD3 against *S. aureus* and *P. aeruginosa* is not affected by NaCl concentrations up to 150 mM, while the peptide is partially inhibited when tested against *E. coli*, at NaCl concentrations higher than 50 mM

Table 2. Potential Advantages and Limits of the Use of HBD3 as a New Antimicrobial Agent

Advantages	Limits
- Strong antibacterial activity	- Partial inhibition by biological fluids
- Broad antimicrobial activity spectrum	- Natural resistance described for a few species
- Active against multidrug-resistant strains	- Potential development of acquired resistance
- Low or no inter-strain variability	- Difficulty in synthesis
- Synergistic with conventional drugs and with other host antimicrobial proteins	
- Spatial arrangement dispensable for antimicrobial activity	
- Relatively salt-insensitive	
- Low toxicity for host cells	
- Possibility to modulate chemotactic activity and/or cytotoxicity without effecting antimicrobial activity	
- Ability to retain antimicrobial activity in biological fluids at concentrations potentially compatible with exogenous administration	
- Capacity to induce adaptive immune responses	

[17]. It has been proposed that the relative insensitiveness of HBD3 to salts might be due to its high net positive charge which allows the peptide to interact with negatively charged bacterial surface overcoming, in a competitive manner, the inhibitory effect of mono/divalent cations [18]. The ability to maintain antimicrobial activity in the presence of salts may represent an additional characteristic crucially important in the case of *in vivo* administration of the peptide.

An important characteristic of AP is that they may also exhibit toxic potential on eukaryotic cells, a fact that raises concern when the use of relatively high doses of such peptides for therapeutic purposes is planned. In contrast to other AP, HBD3 does not exhibit a significant lytic activity on human erythrocytes [11]. No cytotoxicity against human peripheral blood mononuclear cells was also observed at concentrations up to 40 μg/ml [31], although inhibition of proliferation of such cells was reported at higher concentration (100 μg/ml) [69]. Finally, when tested at concentrations up to 50 μg/ml, no cytotoxic effect of HBD3 was observed on normal oral epithelial cells and primary culture of gingival fibroblasts [85]. It is generally assumed that the toxic potential of an AP is connected with the extent of hydrophobicity which allows a more efficient interaction with eukaryotic membranes [58], a fact that has been demonstrated to be true also for HBD3 [19]. Interestingly, Kluver *et al*. [19] could design HBD3 derivatives with high net charge and low hydrophobicity which exhibited potent antimicrobial activity and no significant cytotoxic effects towards eukaryotic cells. The same Authors also demonstrated that HBD3 toxic properties are independent on the secondary structure of the peptide in contrast to what observed for the chemotactic activities that strongly depend on the number and position of disulfide connectivities, as also reported by others [68]. Thus, it seems likely that playing on structural properties (i.e. aminoacid composition, number and posistion of Cys residues etc.) a wide variety of HBD3-derivatives/analogues may be designed exhibiting the right balance among antimicrobial and chemotactic activity, and no toxic potential.

One of the main limits of the use of AP as therapeutic agents is their possible inhibition by biological fluids (Fig. **2**). For this reason, it is of paramount importance to test the activity of peptides in biological fluids to better assess their potential ability to remain active also *in vivo*. Recent studies were conducted in our laboratory to evaluate the bactericidal activity of HBD3 against cariogenic Gram-positive (*S. mutans*) or periodontal Gram-negative pathogens (*A. actinomycetemcomitans*) in a liquid assay containing saliva and/or serum [86]. The results obtained demonstrated that, although partially inhibited, HBD3 retains a rapid bactericidal activity against *S. mutans* both in saliva and serum. Interestingly, in serum alone or in combination with saliva, the peptide was bactericidal at concentrations four and two times lower, respectively than those observed for chlorhexidine, a commonly used oral antiseptic agent. In contrast, HBD3 was strongly inhibited against *A. actinomycetemcomitans* in the presence of saliva, serum or both. The inhibitory effect of serum on HBD3 activity was also confirmed against a number of Gram-positive and Gram-negative nosocomial pathogens [48]. Like in the case for oral pathogens, a stronger inhibitory effect of HBD3 activity by serum was observed against Gram-negative than against Gram-positive bacteria. The bacterial species tested in both studies exhibited comparable susceptibility to the peptide in sodium phosphate buffer, but a marked difference in sensitivity in biological fluids, suggesting that components of serum/saliva may differentially interact with the bacterial surfaces of Gram-positive and Gram-negative bacteria, masking the binding sites for the peptide. It is noteworthy that, although partially inhibited, HBD3 was able to exert a bactericidal activity in biological fluids at concentrations that may potentially be obtained by local delivery *in vivo* [87]. Further studies aimed at the identification of HBD3 derivatives able to retain bactericidal activity but with low affinity for molecular components of biological fluids will help to overcome the partial inhibition of the peptide observed in such fluids.

Another possible limit to the potential use of AP as novel agents to prevent or treat infections regards the existence of microorganisms resistant to their activity [58, 88]. Constitutive mechanisms of resistance rely on inherent properties of an organism and are normally expressed even in the absence of peptide exposure. To date, only few bacterial species have

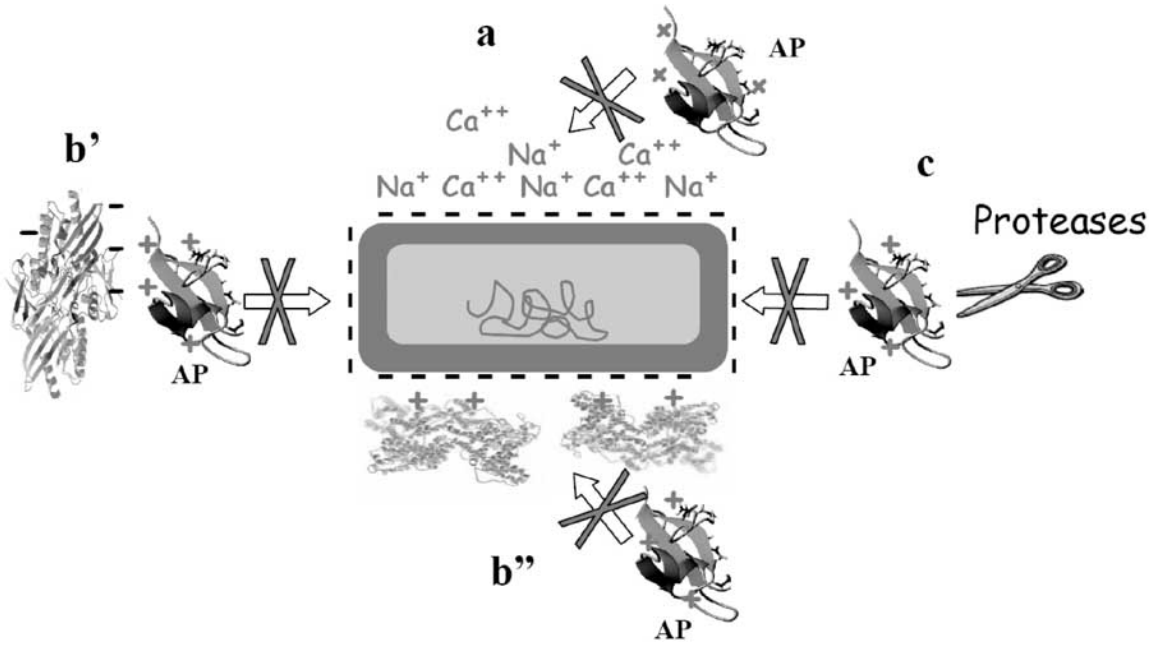

Fig. (2). Schematic illustration of the main inhibitory mechanisms exerted by biological fluids on antimicrobial activity of cationic AP. The high salt concentration found in serum, saliva, or other biological fluids may interfere electrostatically with bacteria/AP interaction (a); proteins or lipoproteins of biological fluids may bind to AP, reducing their bioavailability (b'), or to bacterial surface, masking the binding sites of the peptides (b"); host proteases may digest AP rendering them inactive (c).

been demonstrated to be naturally resistant to HBD3. These include species belonging to the genus *Burkholderia* [45] which are also resistant to HBD1, HBD2, cathelicidins and polymixin B [52, 53, 89], and to the species *Treponema denticola* [90]. Our previous studies [47] indicate that among a number of oral pathogens tested, *P. gingivalis* is the one exhibiting the lowest susceptibility to HBD3, a finding reported also by others [46, 49]. As this microorganism is known to produce a wide range of proteases [91] we recently tested the hypothesis that the relative resistance of *P. gingivalis* to HBD3 is due to its proteolytic activity. Interestingly, we found that stationary-phase culture supernatants of the microorganism are able to degrade synthetic HBD3 within few minutes of incubation and that such a proteolytic activity is mainly due to cys-proteases (manuscript in preparation). Elucidation of the strategies employed by microorganisms to evade antimicrobial host defenses is the prerequisite for the rational design of peptide analogues able to overcome such evasion mechanisms.

Despite the existence of microorganisms naturally resistant to AP, it is generally agreed that, unlike conventional antibiotics, acquisition of resistance by a sensitive microbial strain against AP is extremely improbable [2]. This is likely due to the fact that the main target of AP is the bacterial membrane and that changing the composition and/or the organization of its lipids would not be evolutionary advantageous for a microbial species. Nevertheless, the possible rate of acquisition of AP resistance following their massive introduction in clinical practice is, at the moment, difficult to establish.

The synthesis of defensins is difficult; the main problems concern their purification and systemic delivery, which would be required for *in vivo* studies. The advent of recombinant synthesis technology, which has been successfully utilized for many peptides [92], including HBD3 [11, 93], may help to solve the problem of large-scale production at a relatively low cost. In addition, the observations that HBD3, in contrast to HBD1 and HBD2, may exist in multiple structurally different yet functionally similar forms [68] and that the antimicrobial activity of the peptide seems to be independent by its spatial arrangement [19], may represent advantages when choosing the strategy for large-scale preparation of the peptide.

In conclusion, several characteristics make HBD3 (or its derivatives) an attractive molecule as a potential anti-infective agent. *In vivo* studies to evaluate the therapeutic potential of HBD3 by local and/or systemic applications are desirable.

ACKNOWLEDGEMENT

This work was supported by a grant from "Progetti MIUR", protocol n° 2005062410, Rome, Italy.

ABBREVIATIONS

AP	=	Antimicrobial peptides
HBD1	=	Human beta defensin 1
HBD2	=	Human beta defensin 2
HBD3	=	Human beta defensin 3
HBD4	=	Human beta defensin 4
HBD5	=	Human beta defensin 5
HBD6	=	Human beta defensin 6
IFN-γ	=	Interferon gamma

IL1-β = Interleukin 1 beta

JAK/STAT = Janus kinase/signal transducers and activators of transcription

LPS = Lipopolysaccharide

MBC = Minimum bactericidal concentration

MIC = Minimum inhibitory concentration

MRSA = Methicillin resistant *Staphylococcus aureus*

MSSA = Methicillin sensitive *Staphylococcus aureus*

TNF-α = Tumor necrosis factor alfa

REFERENCES

[1] Zasloff, M. *Nature*, **2002**, *415*, 389.
[2] Hancock, R.E. *Lancet Infect. Dis.*, **2001**, *1*,156.
[3] Harbarth, S.; Samore, M.H. *Emerg. Infect. Dis.*, **2005**, *11*, 794.
[4] Lehrer, R.I. *Nat. Rev. Microbiol.*, **2004**, *2*, 727.
[5] Ganz, T. *Nat. Rev. Immunol.*, **2003**, *3*, 710.
[6] Bensch, K.W.; Raida, M.; Magert, H.J.; Schulz-Knappe, P.; Forssmann, W.G. *FEBS Lett.*, **1995**, *368*, 331.
[7] Harder, J.; Bartels, J.; Christophers, E.; Schroder, J.M. *Nature*, **1997**, *387*, 861.
[8] Zhao, C.; Wang, I.; Lehrer, R.I. *FEBS Lett.*, **1996**, *396*, 319.
[9] Krisanaprakornkit, S.; Weinberg, A.; Perez, C.N.; Dale, B.A. *Infect. Immun.*, **1998**, *66*, 4222.
[10] Harder, J.; Schroder, J.M. *J. Leukoc. Biol.*, **2005**, 77, 476.
[11] Harder, J.; Bartels, J.; Christophers, E.; Schröder, J.M. *J. Biol. Chem*., **2001**, *276*, 5707.
[12] García, J.R.; Jaumann, F.; Schulz, S.; Krause, A.; Rodríguez-Jiménez, J.; Forssmann, U.; Adermann, K.; Klüver, E.; Vogelmeier, C.; Becker, D.; Hedrich, R.; Forssmann, W.G.; Bals, R. *Cell. Tissue Res*., **2001**, *306*, 257.
[13] Jia, H.P.; Schutte, B.C.; Schudy, A.; Linzmeier, R.; Guthmiller, J.M.; Johnson, G.K.; Tack, B.F.; Mitros, J.P.; Rosenthal, A.; Ganz, T.; McCray, P.B.Jr. *Gene*, **2001**, *263*, 211.
[14] Garcia, J.R.; Krause, A.; Schulz, S.; Rodriguez-Jimenez, F.J.; Kluver, E.; Adermann, K.; Forssmann, U.; Frimpong-Boateng, A.; Bals, R.; Forssmann, W.G. *FASEB J*., **2001**, *15*, 1819.
[15] Yamaguchi, Y.; Nagase, T.; Makita, R.; Fukuhara, S.; Tomita, T.; Tominaga, T.; Kurihara, H.; Ouchi, Y. *J. Immunol.*, **2002**, *169*, 2516.
[16] Schutte, B.C.; Mitros, J.P.; Bartlett, J.A.; Walters, J.D.; Jia, H.P.; Welsh, M.J.; Casavant, T.L.; McCray, P.B. Jr. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 2129.
[17] Sahl, H.G.; Pag, U.; Bonness, S.; Wagner, S.; Antcheva, N.; Tossi, A. *J. Leukoc. Biol.*, **2005**, *77*, 1.
[18] Schibli, D.J.; Hunter, H.N.; Aseyev, V.; Starner, T.D.; Wiencek, J.M.; McCray, P.B. Jr.; Tack, B.F.; Vogel, H.J. *J. Biol. Chem.*, **2002**, *277*, 8279.
[19] Kluver, E.; Schulz-Maronde, S.; Scheid, S.; Meyer, B.; Forssmann, W.G.; Adermann, K. *Biochemistry*, **2005**, *44*, 9804.
[20] Fahlgren, A.; Hammarström, S.; Danielsson, A.; Hammarström, M.L. *Clin. Exp. Immunol.*, **2004**, *137*, 379.
[21] McDermott, A.M.; Redfern, R.L.; Zhang, B.; Pei, Y.; Huang, L.; Proske, R.J. *Invest. Ophthalmol. Vis. Sci.*, **2003**, *44*, 1859.
[22] King, A.E.; Fleming, D.C.; Critchley, H.O.D.; Kelly, R.W. *J. Reprod. Immunol*., **2003**, *50*, 1.
[23] Dunsche, A.A.Y.; Dommisch, H.; Siebert, R.; Schröder, J.M.; Jepsen S. *Eur. J. Oral. Sci.*, **2002**, *109*, 121.
[24] Saitoh, M.; Abiko, Y.; Shimabukuro, S.; Kusano, K.; Nishimura, M.; Arakawa, T.; Nakashima, K.; Takuma, T.; Kaku, T.; Igarashi, S. *Arch. Oral Biol.*, **2004**, *49*, 799.
[25] Fang, X.M.; Shu, Q.; Chen, Q.X.; Book, M.; Sahl, H.G.; Hoeft, A.; Stuber, F. *Eur. J. Clin. Invest.*, **2003**, *33*, 82.
[26] Tao, R.; Jurevic, R.J.; Coulton, K.K.; Tsutsui, M.T.; Roberts, M.C.; Kimball, J.R.; Wells, N.; Berndt, J.; Dale, B.A. *Antimicrob. Agents Chemother*., **2005**, *49*, 3888.
[27] Feucht, E.C.; DeSanti, C.L.; Weinberg, A. *Oral Microbiol. Immunol*., **2003**, *18*, 359.
[28] Vankeerberghen, A.; Nuytten, H.; Dierickx, K.; Quirynen, M.; Cassiman, J.J.; Cuppens, H. *J. Periodontol.*, **2005**, *76*, 1293.
[29] Zilbauer, M.; Dorrell, N.; Boughan, P.K.; Harris, A.; Wren, B.W.; Klein, N.J.; Bajaj-Elliott, M. *Infect. Immun*., **2005**, *73*, 7281.
[30] Duits, L.A.; Nibbering, P.H.; van Strijen, E.; Vos, J.B.; Mannesse-Lazeroms, S.P.; van Sterkenburg, M.A.; Hiemstra, P.S. *FEMS Immunol. Med. Microbiol.*, **2003**, *38*, 59.
[31] Quinones-Mateu, M.E.; Lederman, M.M.; Feng, Z.; Chakraborty, B.; Weber, J.; Rangel, H.R.; Marotta, M.L.; Mirza, M.; Jiang, B.; Kiser, P.; Medvik, K.; Sieg, S.F.; Weinberg, A. *AIDS*, **2003**, *17*, F39.
[32] Joly, S.; Organ, C.C.; Johnson, G.K.; McCray, P.B. Jr; Guthmiller, J.M. *Mol. Immunol*., **2005,** *42*, 1073.
[33] Wolk, K.; Kunz, S.; Witte, E.; Friedrich, M.; Asadullah, K.; Sabat, R. *Immunity,* **2004**, *21*, 241.
[34] Sørensen, O.E.; Cowland, J.B.; Theilgaard-Monch, K.; Liu, L.; Ganz, T.; Borregaard, N. *J. Immunol.*, **2003**, *170*, 5583.
[35] Bissell, J.; Joly, S.; Johnson, G.K.; Organ, C.C.; Dawson, D.; McCray, P.B. Jr.; Guthmiller, J.M. *J. Oral Pathol. Med.*, **2004**, *33*, 278.
[36] Fellermann, K.; Wehkamp, J.; Herrlinger, K.R.; Stange, E.F. *Eur. J. Gastroenterol. Hepatol.*, **2003**, *15*, 627.
[37] Wehkamp, J.; Harder, J.; Weichenthal, M.; Mueller, O.; Herrlinger, K.R.; Fellermann, K.; Schroeder, J.M.; Stange, E.F. *Inflamm. Bowel Dis.*, **2003**, *9*, 215.
[38] Islam, D.; Bandholtz, L.; Nilsson, J.; Wigzell, H.; Christensson, B.; Agerberth, B.; Gudmundsson, G. *Nat. Med.*, **2001**, *7*, 180.
[39] Taggart, C.C.; Greene, C.M.; Smith, S.G.; Levine, R.L.; McCray, P.B. Jr.; O'Neill, S.; McElvaney, N.G. *J. Immunol.,* **2003**, *171*, 931.
[40] Jurevic, R.J.; Bai, M.; Chadwick, R.B.; White, T.C.; Dale, B.A. *J. Clin. Microbiol.,* **2003**, *41*, 90.
[41] Hollox, E.J.; Armour, J.A.; Barber, J.C. *Am. J. Hum. Genet.*, **2003**, *73*, 591.
[42] Rawlings, J.S.; Rosler, K.M.; Harrison, D.A. *J. Cell. Sci.*, **2004**, *117*, 1281.
[43] Duits, L.A.; Rademaker, M.; Ravensbergen, B.; van Sterkenburg, M.A.; van Strijen, E.; Hiemstra, P.S.; Nibbering, P.H. *Biochem. Biophys. Res. Commun.*, **2001**, *280*, 522.
[44] Bianchi, M.; Meng, C.; Ivashkiv, L.B. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 9573.
[45] Sahly, H.; Schubert, S.; Harder, J.; Rautenberg, P.; Ullmann, U.; Schroder, J.; Podschun, R. *Antimicrob. Agents Chemother*., **2003**, *47*, 1739.
[46] Joly, S.; Maze, C.; McCray, P.B. Jr.; Guthmiller, J.M. *J. Clin. Microbiol.*, **2004**, *42*, 1024.
[47] Maisetta, G.; Batoni, G.; Esin, S.; Luperini, F.; Pardini, M.; Bottai, D.; Florio, W.; Giuca, M.R.; Gabriele, M.; Campa, M. *Antimicrob. Agents Chemother*., **2003**, *47*, 3349.
[48] Maisetta, G.; Batoni, G.; Esin, S.; Florio, W.; Bottai, D.; Favilli, F.; Campa, M. *Antimicrob. Agents Chemother.*, **2006**, *50*, in press.
[49] Ouhara, K.; Komatsuzawa, H.; Yamada, S.; Shiba, H.; Fujiwara, T.; Ohara, M.; Sayama, K.; Hashimoto, K.; Kurihara, H.; Sugai, M. *J. Antimicrob. Chemother.*, **2005**, *55*, 888.
[50] Guo, L.; Lim, K.B.; Poduje, C.M.; Daniel, M.; Gunn, J.S.; Hackett, M.; Miller, S.I. *Cell*, **1998**, *95*, 189.
[51] Dommisch, H.; Acil, Y.; Dunsche, A.; Winter, J.; Jepsen, S. *Oral Microbiol. Immunol.*, **2005**, *20*, 186.
[52] Saiman, L.; Tabibi, S.; Starner, T.D.; San Gabriel, P.; Winokur, P.L.; Jia, H.P.; McCray, P.B. Jr.; Tack, B.F. *Antimicrob. Agents Chemother*., **2001**, *45*, 2838.
[53] Baird, R.M.; Brown, H.; Smith, A.W.; Watson, M.L. *Immunopharmacology*, **1999,** *44*, 267.
[54] Schutte, B.C.; McCray, P.B. Jr. *Annu. Rev. Physiol.*, **2002**, *64*, 709.
[55] Chen, X.; Niyonsaba, F.; Ushio, H.; Okuda, D.; Nagaoka, I.; Ikeda, S.; Okumura, K.; Ogawa, H. *J. Dermatol. Sci*., **2005**, *40*, 123.
[56] Midorikawa, K.; Ouhara, K.; Komatsuzawa, H.; Kawai, T.; Yamada, S.; Fujiwara, T.; Yamazaki, K.; Sayama, K.; Taubman, M.A.; Kurihara, H.; Hashimoto, K.; Sugai, M. *Infect Immun*., **2003**, *71*, 3730.
[57] Ryley, H.C. *Rev. Med. Microbiol.,* **2001**, *12*, 177.
[58] Yeaman, M.R.; Yount, N.Y. *Pharmacol. Rev*., **2003**, *55*, 27.
[59] Shai, Y. *Biochim. Biophys. Acta*, **1999**, *1462*, 55.
[60] Starner, T.D.; Swords, W.E.; Apicella, M.A.; McCray, P.B. Jr. *Infect. Immun.*, **2002,** *70*, 5287.

[61] Nishi, H.; Komatsuzawa, H.; Fujiwara, T.; McCallum, N.; Sugai, M. *Antimicrob. Agents Chemother.*, **2004**, *48*, 4800.
[62] Peschel, A.; Jack, R.W.; Otto, M.; Collins, L.V.; Staubitz, P.; Nicholson, G.; Kalbacher, H.; Nieuwenhuizen, W.F.; Jung, G.; Tarkowski, A.; van Kessel, K.P.M.; van Strijp J.A.G. *J. Exp. Med.*, **2001**, *193*, 1067.
[63] Peschel, A.; Otto, M.; Jack, R.W.; Kalbacher, H.; Jung, G.; Gotz, F. *J. Biol. Chem.*, **1999**, *274*, 8405.
[64] Vuong, C.; Voyich, M.J.; Fisher, E.R.; Braughton, K.R.; Whitney, A.R.; DeLeo, F.R.; Otto, M. *Cell. Microbiol.*, **2004**, *6*, 269.
[65] Vuong, C.; Kocianova, S.; Voyich, J.M.; Fischer, E.R.; DeLeo, F.R.; Otto, M. *J. Biol. Chem.*, **2004**, *279*, 54881.
[66] Boniotto, M.; Antcheva, N.; Zelezetsky, I.; Tossi, A.; Palumbo, V.; Verga Falzacappa, M.V.; Sgubin, S.; Braida, L.; Amoroso, A.; Crovella, S. *Biochem. J.*, **2003,** *374*, 707.
[67] Hoover, D.M.; Wu, Z.; Tucker, K.; Lu, W.; Lubkowski, J. *Antimicrob. Agents Chemother.*, **2003**, *47*, 2804.
[68] Wu, Z.; Hoover, D.M.; Yang, D.; Boulegue, C.; Santamaria, F.; Oppenheim, J.J.; Lubkowski, J.; Lu, W. *Proc. Natl. Acad. Sci. USA*, **2003**, *100*, 8880.
[69] Sun, L.; Finnegan, C.M.; Kish-Catalone, T.; Blumenthal, R.; Garzino-Demo, P.; La Terra Maggiore, G.M.; Berrone, S.; Kleinman, C.; Wu, Z.; Abdelwahab, S.; Lu, W.; Garzino-Demo, A. *J. Virol.* **2005**,*79*, 14318.
[70] Yang, D.; Chertov, O.; Bykovskaia, S.N.; Chen, Q.; Buffo, M.J.; Shogan, J.; Anderson, M.; Schroder, J.M.; Wang, J.M.; Howard, O.M.; Oppenheim, J.J. *Science*, **1999**, *286*, 525.
[71] Territo, M.C.; Ganz, T.; Selsted, M.E.; Lehrer, R. *J. Clin. Invest.*, **1989**, *84*, 2017.
[72] Murphy, C.J.; Foster, B.A.; Mannis, M.J.; Selsted, M.E.; Reid, T.W. *J. Cell. Physiol.*, **1993**, *155*, 408.
[73] Aarbiou, J.; Ertmann, M.; van Wetering, S.; van Noort, P.; Rook, D.; Rabe, K.F.; Litvinov, S.V.; van Krieken, J.H.; de Boer, W.I.; Hiemstra, P.S. *J. Leukoc. Biol.*, **2002**, *72*, 167.
[74] Van Wetering, S.; Mannesse-Lazeroms, S.P.; Van Sterkenburg, M.A.; Daha, M.R.; Dijkman, J.H.; Hiemstra, P.S. *Am. J. Physiol.*, **1997**, *272*, L888.
[75] Chaly, Y.V.; Paleolog, E.M.; Kolesnikova, T.S.; Tikhonov, II, Petratchenko, E.V.; Voitenok, N.N. *Eur. Cytokine Netw.*, **2000,** *11*, 257.
[76] Niyonsaba, F.; Ushio, H.; Nagaoka, I.; Okumura, K.; Ogawa, H. *J. Immunol.*, **2005**, *175*, 1776.
[77] Befus, A.D.; Mowat, C.; Gilchrist, M.; Hu, J.; Solomon, S.; Bateman, A. *J. Immunol.*, **1999**, *163*, 947.
[78] Yang, D.; Biragyn, A.; Kwak, L.W.; Oppenheim, J.J. *Trends Immunol.*, **2002**, *23*, 291.
[79] Reddy, K.V.; Yedery, R.D.; Aranha C. *Int. J. Antimicrob. Agents*, **2004**, *24*, 536.
[80] Koczulla, A.R.; Bals, R. *Drugs*, **2003**, *63*, 389.
[81] Hancock, R,E.; Patrzykat, A. Curr. *Drug Targets Infect. Disord.*, **2002**, *2*, 79.
[82] Paquette, D.W.; Simpson, D.M.; Friden, P.; Braman, V.; Williams, R.C. *J. Clin. Periodontol.*, **2002**, *29*, 1051.
[83] Levin, M.; Quint, P.A.; Goldstein, B.; Barton, P.; Bradley, J.S.; Shemie, S.D.; Yeh, T.; Kim. S.S.; Cafaro, D.P.; Scannon, P.J.; Giroir. B.P. *Lancet*, **2000**, *356*, 961.
[84] Pristovsek, P.; Kidric, J. *Mini Rev. Med. Chem.*, **2001,** *1*, 409.
[85] Nishimura, M.; Abiko, Y.; Kurashige, Y.; Takeshima, M.; Yamazaki, M.; Kusano, K.; Saitoh, M.; Nakashima, K.; Inoue, T.; Kaku, T. *J. Dermatol. Sci.*, **2004**, *36*, 87.
[86] Maisetta, G.; Batoni, G.; Esin, S.; Raco, G.; Bottai, D.; Favilli, F.; Florio, W.; Campa, M. *Antimicrob. Agents Chemother.*, **2005,** *49*, 1245.
[87] Mosca, D.A.; Hurst, M.A.; So, W.; Viajar, B.S.; Fujii, C.A.; Falla, T.J. *Antimicrob. Agents Chemother.*, **2000**, *44*, 1803.
[88] Peschel, A. *Trends Microbiol.*, **2002**, *10*, 179.
[89] Moore, R.A.; Hancock, R.E. *Antimicrob. Agents Chemother.*, **1986**, *30*, 923.
[90] Brissette, C.A.; Lukehart, S.A. *Infect. Immun.*, **2002**, *70*, 3982.
[91] Kadowaki, T.; Nakayama, K.; Okamoto, K.; Abe, N.; Baba, A.; Shi, Y.; Ratnayake, D.B.; Yamamoto, K. *J. Biochem.*, **2000**, *128*, 153.
[92] Chen, H.; Xu, Z.; Peng, L.; Fang, X.; Yin, X.; Xu, N.; Cen, P. *Peptides*, **2005**, Oct 11; [Epub ahead of print].
[93] Chen, S.; He, F.T.; Dong, Y.L.; Li, R.F.; Gao, H.G.; Chen, M.; Peng, J.H. *Sheng Wu Gong Cheng Xue Bao*, **2004**, *20*, 490.

Received: January 30, 2006 **Revised: April 12, 2006** **Accepted: April 13, 2006**