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Defensins as anti-inflammatory compounds and mucosal adjuvants

Karl G Kohlgraf,

Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA 52242, USA, Tel.: +1 319 353 4282, Fax: +1 319 335 8895, karl-kohlgraf@uiowa.edu

Lindsey C Pingel,

Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA 52242, USA, Tel.: +1 319 335 8077, Fax: +1 319 335 8895, lindsey-pingel@uiowa.edu

Deborah E Dietrich, and

Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA 52242, USA, Tel.: +1 319 335 8077, Fax: +1 319 335 8895, deborah-dietrich@uiowa.edu

Kim A Brogden[†]

Dows Institute for Dental Research and Department of Periodontics, College of, Dentistry, The University of Iowa, Iowa City, IA 52242, USA, Tel.: +1 319 335 8077, Fax: +1 319 335 8895, kim-brogden@uiowa.edu

Abstract

Human neutrophil peptide α -defensins and human β -defensins are small, well-characterized peptides with broad antimicrobial activities. In mixtures with microbial antigens, defensins attenuate proinflammatory cytokine responses by dendritic cells in culture, attenuate proinflammatory cytokine responses in the nasal fluids of exposed mice and enhance antibody responses in the serum of vaccinated mice. Although the exact mechanisms are unknown, defensins first start by binding to microbial antigens and adhesins, often attenuating toxic or inflammatory-inducing capacities. Binding is not generic; it appears to be both defensin-specific and antigen-specific with high affinities. Binding of defensins to antigens may, in turn, alter the interaction of antigens with epithelial cells and antigen-presenting cells attenuating the production of proinflammatory cytokines. The binding of defensins to antigens may also facilitate the delivery of bound antigen to antigen-presenting cells in some cases via specific receptors. These interactions enhance the immunogenicity of the bound antigen in an adjuvant-like fashion. Future research will determine the extent to which defensins can suppress early events in inflammation and enhance systemic antibody responses, a very recent and exciting concept that could be exploited to develop therapeutics to prevent or treat a variety of oral mucosal infections, particularly where inflammation plays a role in the pathogenesis of disease and its long-term sequelae.

Keywords

adjuvant; defensin; innate immunity; mucosal immunity; oral inflammation

[†]Author for correspondence: Department of Periodontics and Dows Institute for Dental Research, College of Dentistry, The University of Iowa, Iowa City, IA 52242, USA, Tel.: +1 319 335 8077, Fax: +1 319 335 8895, kim-brogden@uiowa.edu.

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Vast microbial burden in the oronasal cavity

Mucosal surfaces of the oronasal cavity are continually exposed to microbial antigens in respirable air, microbial antigens in ready-to-eat and processed foods, potable water, microbial antigens from high concentrations of commensal flora and even microbial antigens from low concentrations of opportunistic pathogens [1,2]. In the oral cavity alone, the microbial community contains 500–700 phylotypes from both the Eubacteria and Archaeobacteria domains [3,4]. Throughout the oronasal cavity, phylotypes occur in site-specific areas with a different prevalence of microorganisms in the nasopharynx, oropharynx, gingival crevice, dental plaque, tongue and saliva [5,6].

Surprisingly, in spite of the microbial burden, there is minimal inflammation in oral and nasal tissues under normal conditions in healthy individuals, an observation made previously by Soderblom *et al.* [7]. This observation suggests that local innate immune mechanisms in mucosal secretions keep continual proinflammatory cytokine release and unrestricted mucosal inflammation ‘in check.’ It also suggests that the mucosal adaptive immune response recognizes, processes and manages microbial oronasal infection and colonization [8–10]. These mechanisms may involve human neutrophil peptide α -defensins (HNPs) and human β -defensins (HBDs). Such a process may start with the induction of defensins by microorganisms or their byproducts (Figure 1). These defensins, in mucosal secretions, are then available to bind to microorganisms or their byproducts. By binding, the defensin alters the ability of the antigen to attach to host cells, alters signal transduction pathways and, thus, attenuates the production of proinflammatory cytokine responses. The defensin and antigen complexes may be more readily taken up by antigen-presenting cells, which enhances the production of antigen-specific antibody responses, a concept proposed as the ‘dead microbe defensin’ or ‘defensin antigen’ by Yang and colleagues [11,12]. Complexes may be subject to defensin receptor-mediated internalization and, thus, deliver microbial antigens to immature dendritic cells more efficiently.

This article looks beyond the role of defensins as mucosal antimicrobial agents and focuses on their ability to suppress the production of proinflammatory cytokines and enhance the production of antigen-specific antibodies. The types and characteristics of defensins found on oronasal surfaces are reviewed and their ability to bind to microbial antigens, attenuate proinflammatory cytokine responses and act as adjuvants to potentiate a systemic antibody response to mucosal administered antigen–defensin mixtures is discussed. Additional information can be found in the comprehensive reviews by Yang and colleagues [11–13]. Finally, this article presents the concept that defensins could serve as pharmaceuticals to improve therapies to treat and control a wide variety of oral mucosal infections and inflammatory disorders.

Defensins

The HNPs, HBDs and human θ -defensins [14] are small, host-derived peptides with a variety of innate and adaptive immune functions [15–20]. Although related, these peptides have distinct differences in their cysteine amino acid composition motifs and disulfide bonding orders, differences in individual peptide amino acid compositions, differences in masses and differences in isoelectric points (Table 1).

Human neutrophil peptide α -defensins are abundantly produced in the oronasal cavities. They are expressed in oral tissues and salivary glands and are present in saliva, gingival crevicular fluid and nasal secretions (Table 2). HNP1–3 are found in neutrophil granules, monocytes and natural killer cells; HNP4 is found in primary neutrophil azurophil granules; and human α -defensin (HD)5 and 6 are found in mucosal Paneth cells. HNPs contain 29–35 amino acid

residues and are very similar in size and amino acid composition [17,18]. HNP1 has an additional N-terminal alanine residue and HNP3 has an additional N-terminal aspartic acid residue. HNP4 is slightly larger in size, more variable in its amino acid composition, rich in arginine (15.2 mol%) and significantly more hydrophobic.

Human β -defensins are also abundantly produced in the oronasal cavities. They, too, are expressed in oral tissues and are present in saliva, gingival crevicular fluid, nasal secretions and bronchoalveolar lavage fluids (Table 2). HBD1 contains 36 amino acid residues with a +5 charge, HBD2 contains 41 amino acid residues with a +7 charge, and HBD3 contains 45 amino acid residues, has a β -sheet structure in solution, forms dimers and has a net +11 charge. Interestingly, high levels of HBD3 mRNA expression are seen in healthy tissues [21].

Defensins were originally discovered because of their antimicrobial activities and early interest centered on their ability to kill Gram-negative bacteria, Gram-positive bacteria, fungi and inhibit some viral infections [22–24]. More recent interest centered on their profound effect on the immune system [11,17,18]. Defensins enhance phagocytosis by macrophages, chemoattract monocytes, macrophages, T lymphocytes, mast cells and immature dendritic cells, induce the production of proinflammatory cytokines, suppress the production of proinflammatory cytokines to select microbial antigens, activate and degranulate mast cells, regulate the complement system, inhibit glucocorticoid production and enhance antigen-specific immune responses.

Defensins are induced by a variety of stimuli and ligands through a number of signaling pathways. Microorganisms or their byproducts induce defensins via Toll-like receptor (TLR)-dependent and -independent signaling pathways [25]. For example, TLR2 and 4 induce expression of HBD2 by lipopolysaccharide (LPS) or peptidoglycan via JNK pathways [26, 27]; TLR5 induces expression of HBD2 by *Salmonella enteritidis* flagellin by p38 and ERK pathways [28]; and expression of HBD2 by *Fusobacterium nucleatum* in human gingival epithelial cells is mediated by p38 and JNK pathways [29]. Cytokines and chemokines also induce the expression of defensins. For example, TNF- α /IFN- γ induces HBD2 and HBD3 by activating signal transducer and activator of transcription (STAT)1 and NF- κ B signaling pathway [30] and IL-4 and IL-13 activate STAT-6 and induce suppressor of cytokine signaling (SOCS)1 and SOCS3. This interferes with STAT-1 and NF- κ B signaling, thereby inhibiting TNF- α /IFN- γ -mediated induction of HBD2 and HBD3, suggesting that targeting the STAT-1-signaling pathway or SOCS expression enhances β -defensin expression. The NF- κ B pathway does not appear to play a prominent role in inducing the expression of HBD3 [15]. Induction of HBD3 appears to be mediated by the p38 pathway [31] and EGF receptor/ERK pathway [32,33].

In turn, defensins are capable ligands and can activate cells via a variety of signaling pathways. For example, mouse β -defensin (MBD)2 activates dendritic cells via the TLR4-dependent pathway [34]; HBD2 activates the 70-kDa ribosomal S6 kinase (p70S6K) pathway [35]; HBD2 induces CaCo-2 intestinal cells to downregulate mRNA for TLR7, IL-1R-associated kinase and IL-8, and upregulate NF- κ Bp65 [36]; HBD2 induces MCF-7 breast epithelial cells to upregulate mRNA for inhibitor of κ B α , NF- κ Bp65, Tollip, MyD88, IL-1R-associated kinase and TLR7 [36]. HBDs also induce phosphorylation of EGF receptor, STAT1 and 3, and production of IL-18 through p38 and ERK1/2 MAPK pathways [37].

Defensins attenuate proinflammatory cytokine responses

Defensins clearly regulate innate immune responses. They have direct antimicrobial activity, chemoattract phagocytic and mast cells, induce inflammatory mediators, regulate the functions of phagocytes and regulate the complement system. For more detailed information, see the comprehensive reviews by Yang and colleagues [11–13].

More important to this article, HNPs and HBDs also regulate early inflammatory events. Very recent and independent reports show that HNPs and HBDs have powerful anti-inflammatory effects on human monocytes [38], human monocyte-derived macrophages [39] and human myeloid dendritic cells [40]. A comparison of the proinflammatory cytokine responses attenuated by HNPs and HBDs in these cells treated with LPS or *Porphyromonas gingivalis* recombinant hemagglutinin B (rHagB) is shown in Table 3. Shi *et al.* found that HNP1 blocked the release of IL-1 β from LPS-activated monocytes, but not the expression and release of TNF- α [38]. Miles *et al.* found that human neutrophils dying by apoptosis or necrosis release HNPs that inhibit the secretion of multiple proinflammatory cytokines from macrophages [39]. HNPs are the active anti-inflammatory factor released by apoptotic/necrotic neutrophils, HNPs inhibit the proinflammatory cytokine production by macrophages in the presence of both live and dead whole bacteria, HNPs inhibit the LPS-mediated activation of macrophages and HNPs do not affect the release of proinflammatory cytokines (already in secretory vesicles) from macrophages. Pingel *et al.* found that HBD3 attenuates the IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF- α responses of human myeloid dendritic cells to rHagB [40].

An initial step in a defensin-attenuated proinflammatory cytokine response is the likely binding of defensins in mucosal secretions to microorganisms or their byproducts [41]. Subsequent studies have clearly shown that defensins bind to microbial membranes [42,43], surface adhesins [40,44], LPS [42,45] and select bacterial toxins [46–48]. Binding is both defensin specific and antigen specific. Interestingly, HNP1, HNP2 and HBD3 bind to microbial products and viruses more readily than HBD1 and HBD2.

As an early and desirable outcome, binding of defensins to microbial products often attenuates their toxic or inflammatory-inducing capacities. For example, a synthetic peptide comprising the core region of HBD23 (e.g., defensin- β [DEFB]123) prevents LPS-induced production of TNF- α secretion in murine monocyte RAW264.7 cells, abolishes LPS-mediated MAPK induction in RAW264.7 cells, protects mice against LPS-mediated acute sepsis and prevents LPS-induced mortality in C57BL/6 mice [45]. HNP1 binds to *Bacillus anthracis* lethal factor, causes a conformational change that prevents enzymic conversion, and protects mice from *B. anthracis* lethal factor intoxication and death [47]. Rhesus θ -defensins 1–3 also bind to *B. anthracis* lethal factor [49]. HNP1, HNP3 and HD5, but not HBD1, bind with affinity to *Clostridium difficile* toxin B and inhibit toxin B-catalyzed *in vitro* glucosylation of Rho guanosine triphosphatases [46].

As shown by Lehrer and colleagues, defensins also bind to a variety of viruses and viral products (Table 4). HNP1–4, HD5, HD6 and HBD3 inhibit herpes simplex virus infection, but HBD1 and HBD2 do not [23]. HNP4 and HD6 prevent virus attachment and entry into cells, whereas HNP1–3 and HD5 only inhibit postentry events [23]. Using surface plasmon resonance spectroscopic analysis, defensins interact differently with herpes simplex virus glycoprotein B and heparan sulfate, its receptor [23]. HNP1, HNP2, HNP3 and HD5 bind to glycoprotein B with high affinity but bind to heparan sulfate with low affinity; HNP4 and HD6 bind to heparan sulfate, but not to glycoprotein B; HBD3 binds to both glycoprotein B and heparan sulfate; and HBD1 and HBD2 do not bind to either glycoprotein B or heparan sulfate. Retrocyclin-1 binds to CD4 (the primary host receptor for HIV-1), to galactosylce-ramide (an alternative cell-surface receptor for HIV-1), to gp120 (the envelope glycoprotein of HIV-1) [50–52] and to the ectodomain of gp41 (HIV-1) [53].

Surface plasmon resonance spectroscopy has also enabled us to show differential binding of HNPs and HBDs to immobilized rHagB and recombinant fimbrillin (rFimA), which are nonfimbrial and fimbrial adhesins, respectively, from the oral periodontal pathogen *P. gingivalis* [40,44]. When the results for each ligand are compared, HNPs and HBDs have

different binding recognition rates, different dissociation rates and different affinities (Table 4) for these bacterial adhesins. Similar to the trends above, HBD3, HNP1 and HNP2 (in decreasing order) preferentially bind to immobilized rHagB, and HNP1, HNP2 and HBD3 (in decreasing order) preferentially bind to immobilized rFimA. Interestingly, HBD1 and 2 generally had significantly lower response unit signal responses than HNP2, HNP1 and HBD3, yet still bound with high affinities.

The binding of HNPs and HBDs to microbial antigens and adhesins has implications on how these microbial products interact with eukaryotic cellular surfaces and receptors. There is some evidence that the binding of defensins to microbial antigens and adhesins alters their binding to eukaryotic cells. Recently, we assessed whether HBD3 alters the binding of rHagB to cell membrane surfaces [54]. In a pilot study, we hypothesized that HBD3 alters rHagB binding to keratinocytes and human myeloid dendritic cells. To test this, human telomerase reverse transcriptase (hTERT)-immortalized oral keratinocytes and human myeloid dendritic cells were exposed to rHagB (0.1 μ M), HBD3 + rHagB (10:1 molar ratio), HBD3 (1.0 μ M) or 0.01 M phosphate-buffered saline (PBS), pH 7.2. Cells were washed, fixed in 4% paraformaldehyde, treated with monoclonal antibody to rHagB, treated with fluorescence antimouse antibody and topro3 (for nuclear staining), and examined using confocal microscopy. Keratinocytes and dendritic cells exposed to rHagB had surface fluorescence, suggesting that rHagB was binding to cell membranes (Figure 2). Fluorescence was diminished in keratinocytes and dendritic cells incubated with HBD3 + rHagB and absent in cells incubated with HBD3 or PBS (not shown).

In another pilot study, we hypothesized that rHagB can be detected on the surface of human myeloid dendritic cells by protein A-colloidal gold immunoelectron microscopy and that prior incubation of HBD3 with rHagB reduces this response [55]. To test this, human myeloid dendritic cells were exposed to rHagB (0.1 μ M), HBD3 + rHagB (10:1 molar ratio), HBD3 (1.0 μ M) or 0.01 M PBS, pH 7.2. Cells were fixed with glutaraldehyde and processed for ultramicrotomy. Sections were etched with sodium metaperiodate, incubated with monoclonal antibody to rHagB, incubated with protein A-colloidal gold, stained with uranyl acetate and lead citrate and examined by transmission electron microscopy. Dendritic cells incubated with rHagB had protein A-colloidal gold label at the dendritic cell surface and throughout the cell cytoplasm, suggesting that rHagB binds to the human myeloid dendritic cell surface and is rapidly processed by the cell. Dendritic cells incubated with HBD3 + rHagB had less protein A-colloidal gold label at the cell surface, suggesting that rHagB binding can be altered by prior incubation with HBD3 (Figure 3).

If the binding of defensins to adhesins simply neutralizes their ability to bind to cellular surfaces and receptors then one would expect that the inhibition of binding would not activate signal transduction pathways or induce the production of proinflammatory cytokines and chemokines. It appears that the binding of defensins to adhesins alters their ability to bind to cellular receptors; the production of proinflammatory cytokines is attenuated but the production of chemokines is not [40]. Human myeloid dendritic cells incubated with rHagB produce proinflammatory cytokines (IL-6, GM-CSF, TNF- α and IL-12p40), IL-10 and the chemokines IL-8 (CXCL8), IFN-inducible protein (IP)-10 (CXCL10), monocyte chemoattractant protein (MCP)-1 (CCL2), macrophage inflammatory protein (MIP)-1 α (CCL3) and RANTES (CCL5). HBD3 only attenuates the IL-6, IL-10, GM-CSF and TNF- α responses induced by rHagB (Table 3).

In mice, HNPs and HBDs attenuate proinflammatory cytokine responses to rHagB or rFimA [56]. To show this, C57BL/6 mice were given rHagB without and with HNP1, HNP2, HBD1, HBD2 or HBD3. At 24 h, all mice were euthanized and cytokine concentrations were determined in saliva, nasal wash fluids, bronchoalveolar lavage fluids and serum. At 24 h after

exposure, rHagB induces IL-6 and keratinocyte-derived chemokine (KC) responses in the nasal wash fluid, which were attenuated by HNP2, HBD1 and HBD3.

If the binding of defensins to *P. gingivalis* adhesins alters the binding of adhesins to cellular receptors and attenuates the production of proinflammatory cytokines, then one would expect that this event would also alter the ability of adhesins to trigger signal transduction pathways. This appears to be the case where the binding of HBD3 to rHagB selectively alters the ability of rHagB to induce MAPK signaling pathways [40]. The MAPK pathways are important in controlling the type and magnitude of the inflammatory response to *P. gingivalis* and its extracellular products [57]. rHagB induces p38, JNK1/2, and ERK1/2 responses in human myeloid dendritic cell lysates within 10–30 min. At 30 min, HBD3/rHagB mixtures in human myeloid dendritic cells induce lower signals of phosphorylated ERK1/2 but not signals of phosphorylated JNK1/2 or p38 [40].

Defensins enhance adaptive immunity

Many antimicrobial peptides have the properties of classical adjuvants and elicit enhanced humoral and/or cellular immune responses against tumor antigens, protein antigens and even microbial antigens [41,58]. Enhanced immune responses occur with mixtures (containing antimicrobial peptides and antigens), conjugates (containing antimicrobial peptide coupled to antigens) and fusion proteins (containing expressed antimicrobial peptides and antigens).

Adjuvants are a broad class of compounds that, when administered with an antigen, increase the antigenicity/immunogenicity of the antigen and induce humoral and/or cell-mediated immune responses. By definition, they must not induce harmful side effects that include hypersensitivity or autoimmunity, contain antigens that cross-react with human tissue, contain contaminants that induce harmful side effects or be nonbiodegradable. The need for effective adjuvants spurred intense research efforts in the 1970s to identify and characterize components that have superior immunopotentiating activities without undesired side effects.

A large diversity of substances increase the host immune response to poorly immunogenic antigens, recombinant proteins, synthetic antigens, and conjugated antigens. For a comprehensive list of adjuvants, see the review articles by Pingel *et al.* [58] and Guy [59]. These substances include: nonbacterial adjuvants containing minerals, synthetic polymers, polyanions, or hydrophobic or amphipathic substances [60–62]; bacterial adjuvants consisting of whole bacteria, LPS, peptidoglycan fragments, DNA, toxins or other cellular components from Gram-positive bacteria, Gram-negative bacteria or *Mycobacteria* species [63–66]; and cytokines, costimulatory agonists, and antimicrobial peptides and proteins [67–70]. In the latter group, lactoferrin is an important mediator in host defense and a potential immunological adjuvant [71,72]. Cathelin-related antimicrobial peptide (CRAMP) induces humoral and cellular antigen-specific immune responses in mice to ovalbumin in a dose-dependent manner [73]. Melittin from the honey bee *Apis mellifera*, coadministered intranasally with tetanus toxoid, induces higher antibody titers in BALB/c mice in comparison to that induced in mice receiving tetanus toxoid alone [74]. The artificial cationic antimicrobial peptide, KLKLLLLLKLK, induces high levels of antigen-specific antibodies to ovalbumin or a commercially available influenza vaccine [75]. LL-37 enhances an antitumor immune response of macrophage colony-stimulating factor receptor (M-CSFR)_{J6-1}, a potential target for tumor immunotherapy, when LL-37 is genetically fused with M-CSFR_{J6-1} and administered to mice [76]. Anti-M-CSFR antibody or M-CSF-soluble receptor inhibits the growth of leukemia and hepatoma cell lines overexpressing M-CSF and M-CSFR.

It is not surprising that defensins, too, elicit enhanced humoral, protective and therapeutic immune responses (Table 5). They interact with G protein-coupled receptors on immature dendritic cells, stimulate dendritic cell maturation and have direct effects on T lymphocytes.

For more detailed information, see the comprehensive reviews by Yang and colleagues [11–13].

Defensins enhance antitumor immune responses [77–79]. MBD-based vaccines elicit potent cell-mediated responses and antitumor immunity when genetically fused with another nonimmunogenic tumor antigen [77]. The fusion protein, consisting of MBD linked to a tumor antigen, acts directly on immature dendritic cells as an endogenous ligand for TLR4 and upregulates costimulatory molecules, induces dendritic-cell maturation and induces the production of lymphokines [77]. This likely promotes T-cell-dependent cellular immunity and antigen-specific antibody production. For example, mice immunized with 1–2 µg plasmid DNA encoding fusion constructs of MBD2 and 3 with either lymphoma-specific VH and VL fragments from 38C13 cells (called sFv38) or A20 cells (called sFv20) elicit differential humoral, protective and therapeutic immune responses against two different syngeneic lymphomas [77]. First, fusion proteins MBD2–sFv38, MBD3–sFv38, MBD2–sFv20 and MBD3–sFv20 all induce IgG1 antibody responses but fusion proteins containing MBD3 induce higher antibody responses than fusion proteins containing MBD2. The antibody responses were comparable to that induced by vaccination with tumor-derived intact Ig protein conjugated to keyhole limpet hemocyanin (KLH). Second, fusion proteins MBD2–sFv38 and MBD3–sFv38 protect mice against a 20-fold lethal dose of syngeneic tumor. Again, the response was comparable to that induced by vaccination with Ig conjugated to KLH. Third, fusion protein MBD2–sFv20 could rescue tumor-bearing mice whereas fusion proteins MBD2–sFv38 and MBD3–sFv20 could not. Although the fusion proteins MBD3–sFv38 and MBD3–sFv20 induce higher antibody responses than fusion proteins MBD2–sFv38 and MBD2–sFv20, fusion protein MBD3–sFv20 could not rescue tumor-bearing mice. Biragyn *et al.* point out that induction of humoral immunity is not sufficient to eradicate A20 B-cell lymphoma and that fusions with MBD2 induce specific cellular antitumor responses.

Ma *et al.* assess the antileukemia activity of MBD2 in a murine model of acute lymphoid leukemia by using a modified leukemia cell (e.g., L1210 cell line) vaccine designed to secrete biologically functional MBD2 (called MBD2-L1210) [79]. Mice inoculated with MBD2-L1210 have enhanced cytotoxic T-lymphocyte and natural-killer activity and augments IL-12 and IFN-γ production, which protects them from lethal challenge with L1210 cells.

Defensins enhance adaptive immune responses to coadministered protein antigens [78,80,81]. Intranasal delivery of HNPs with ovalbumin or intraperitoneal administration of defensins with KLH both enhance adaptive immune responses *in vivo*. Mice, immunized with KLH adsorbed to aluminum hydroxide with defensins, produce significantly higher amounts of KLH-specific IgG1, IgG2a and IgG2b antibodies at 14 days after immunization. The splenic KLH-specific proliferative responses are higher in mice treated with KLH and defensins than in those treated with KLH alone [78].

Lillard and colleagues showed that HNP1–3 enhance systemic IgG, but not IgA, antibody responses with the help of CD4⁺ Th1- and Th2-type cytokines [80]. Intranasal delivery of HNP1–3 with ovalbumin in mice enhances ovalbumin-specific serum IgG antibody responses. Interestingly, ovalbumin-specific IgA antibody responses were not induced in mucosal secretions or in serum. Ovalbumin-specific IgM and IgG antibodies were induced in serum, the latter characterized by IgG1, IgG2b and IgG2a. CD4⁺ T cells displayed higher ovalbumin-specific proliferative responses and elevated production of IFN-γ, IL-5, IL-6 and IL-10 when compared with control mice receiving ovalbumin alone. HNP1–3 also enhanced proliferative responses and Th cytokine secretion profiles of CD3ε-stimulated spleen- and Peyer's patch-derived naive CD4⁺ T cells. HNP1–3 modulated the expression of costimulatory molecules by LPS- or CD3ε-stimulated splenic and Peyer's patch B- or T-cell populations, respectively.

Human β -defensins, coadministered intranasally with ovalbumin, also induce similar immune responses in mice [81]. Intranasal delivery of HBD1 and HBD2 with ovalbumin in mice also enhances ovalbumin-specific serum IgG antibody responses. HBD1 induces higher ovalbumin-specific serum IgG antibody responses and higher serum IgM antibody responses. HBD2 induces higher ovalbumin-specific serum IgG and lower serum IgM antibody responses. The elevated serum IgG subclasses contains IgG1 and IgG2b. In ovalbumin-stimulated splenic lymphoid cell cultures from immunized mice HBD1 induces higher amounts of IL-10 and HBD2 induces lower amounts of IFN- γ .

In a recent study by Kohlgraf and colleagues, HNPs and HBDs enhance antibody responses to coadministered microbial antigens, rHagB and rFimA from *P. gingivalis* strain 381 [56]. Interestingly, HNPs and HBDs enhance antibody responses to rHagB and, to a lesser extent, rFimA, suggesting that microbial antigen composition may be important. At 21 days after immunization, rHagB without or with HNPs or HBDs did not induce significant IgA or IgM antibody responses in saliva, nasal wash fluid or bronchoalveolar lavage fluid. Mice immunized with rHagB and HNP1 or HBD3 had higher IgG serum antibody responses. In these studies, rFimA was not as immunogenic via the intranasal route and rFimA without and with HNPs or HBDs did not induce significant IgA or IgM antibody responses in saliva, nasal wash fluid, bronchoalveolar lavage fluid or serum. Mice immunized with rFimA and HNP2 had a higher IgG antibody response. These results suggest that HNP1, HNP2 or HBD3, but not HBD1 or HBD2, enhance antibody responses to rHagB, but not rFimA, of *P. gingivalis* in mice.

Defensins as pharmaceuticals

Defensins, expressed in epithelia of many organs and in nonepithelial tissues, protect these tissues from infection, prevent inflammation and promote adaptive immune responses. In some cases, defensin deficiencies are thought to be key factors in the pathogenesis of infection and inflammation through a compromise of innate immunity [82,83]. Although early, it is tempting to speculate that polymorphisms in defensins or differences in copy number decreases their ability to protect these sites and increases the susceptibility of those individuals to infection and inflammation. Copy-number polymorphisms and expression level variations of the HNPs [84] and HBDs [85] may lead to different defensin profiles and possibly correlate with susceptibility to bacterial infections [86,87], Crohn's disease [82,88], periodontal disease [89] and cystic fibrosis [90]. With HBD3, a three copy-number variation was the most frequent genotype occurring in 65.9% of 44 subjects, a two copy-number variation in 30.5% of 44 subjects and a four copy-number variation in 13.6% of 44 subjects [91].

It is tempting to speculate that defensins, defensin fragments or synthetic analogs, like those generated by Dhople and colleagues [42], could serve as therapeutics to treat and control a wide variety of oral mucosal infections and inflammatory disorders. Peptides, particularly those that attenuate proinflammatory cytokine production, could be added to mucoadhesive polymers or buccoadhesive drug delivery systems and used in situations where inflammation plays a role in the pathogenesis of disease.

Alternate strategies, as suggested recently by Diamond and colleagues [17], could involve the use of exogenous modifiers of defensin expression. Since defensins are regulated at the transcriptional level, use of secretagogues could locally increase levels of defensin expression within their proper milieu.

Conclusion

Human neutrophil peptide α -defensins and HBDs are abundantly produced in oral tissues and salivary glands and are present in saliva, gingival crevicular fluid and nasal secretions. Here

they have direct antimicrobial activity, chemoattract phagocytic and mast cells, induce inflammatory mediators, regulate the functions of phagocytes and regulate the complement system. In addition, HNPs and HBDs regulate early inflammatory events and enhance adaptive immunity. HNPs and HBDs have powerful, anti-inflammatory effects on monocytes, macrophages and dendritic cells. They also attenuate early inflammatory events in mouse models of inflammation. Defensins, too, can elicit enhanced humoral, protective and therapeutic antitumor immune responses, enhance adaptive immune responses to coadministered protein antigens, and enhance antibody responses to coadministered microbial antigens. Future research will determine whether defensins or defensin congeners have the ability to serve as pharmaceuticals to improve therapies to treat and control a wide variety of oral mucosal infections and inflammatory disorders.

Future perspective

Ongoing work will identify the rHagB receptor on human dendritic cells, the molecular mechanism by which HBD3 binds to rHagB and the molecular mechanism attenuating the signaling pathway response induced by the HBD3 and rHagB 'complex.' Additional work is needed to assess how polymorphisms affect the ability of HNPs or HBDs to regulate an inflammatory response. Together, these results will lead to a better understanding of the early mechanisms of mucosal inflammation and potentially provide a variety of new therapeutic avenues for treatment and prevention of inflammatory responses.

Executive summary

- Human neutrophil peptide α -defensins (HNPs) and human β -defensins (HBDs) are abundant in oral gingival tissues, salivary glands, salivary secretions and gingival crevicular fluid. Here, they are ideally positioned to interact with an extensive and diverse group of microorganisms and microbial antigens entering or already present in the oral and nasal cavities.
- HNP1, HNP2 and HBD3 generally bind to microbial products and viruses more readily than HBD1 and HBD2. Binding of defensins to microbial products often attenuates their toxic or inflammatory-inducing capacities.
- Binding of defensins to microorganisms or their byproducts alters the ability of these products to bind to eukaryotic cells.
- Binding of defensins to microbial products attenuates their ability to induce the production of proinflammatory cytokines in dendritic cells *in vitro*. Interestingly, the production of proinflammatory cytokines is attenuated but the production of chemokines is not attenuated.
- Binding of defensins to microbial products also attenuates their ability to induce the production of proinflammatory cytokines in the nasal wash fluids of mice.
- The MAPK pathways are important in controlling the type and magnitude of the inflammatory response to *Porphyromonas gingivalis* and its extracellular products. HBD3 + recombinant hemagglutinin B mixtures in human myeloid dendritic cells induces lower signals of phosphorylated ERK1/2 but not signals of phosphorylated JNK1/2 or p38.
- The binding of defensins to antigen promotes adaptive antitumor responses (tumor antigens) and enhances serum antibody responses (ovalbumin and microbial adhesins).

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Figure 1. Defensin-mediated events on the mucosal surface using *Porphyromonas gingivalis* as an example

(1) *P. gingivalis* on the oral gingival epithelium, gingival margin, oral sulcular epithelium or in the enamel space induces the local production of defensins and cytokines. (2) Induced cytokines recruit polymorphonuclear leukocytes to the local site of infection. (3) Together, gingival epithelium and polymorphonuclear leukocytes provide HNPs and HBDs. (4) These defensins bind to microbial antigens and adhesins, creating a complex that alters the binding of the microbial product to epithelial cells and dendritic cells. (5) This interaction with microbial byproducts attenuates local proinflammatory cytokine responses from dendritic cells, thus minimizing inflammation. (6) Dendritic cells in the vicinity take up microbial

product and defensin complexes, then process and present these antigens to naive T cells to enhance antibody responses. HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin.

Figure 2. Human β -defensin 3 alters the binding of recombinant hemagglutinin B to keratinocytes and human myeloid dendritic cells via confocal microscopy

Keratinocytes and human myeloid dendritic cells incubated with rHagB (**A & D**) and then monoclonal antibody to rHagB (followed by fluorescent antimouse antibody) have green fluorescence on the cell surface (arrows). Keratinocytes and human myeloid dendritic cells incubated with HBD3 + rHagB (**B & E**) and then monoclonal antibody to rHagB (followed by fluorescent antimouse antibody) do not have fluorescence on the cell surfaces and only stained nuclei are seen. Nuclei are faint (**E**). Keratinocytes and human myeloid dendritic cells incubated with HBD3 (**C & F**) or PBS (not shown) and then monoclonal antibody to rHagB (followed by fluorescent antimouse antibody) do not have fluorescence on the cell surfaces and only stained nuclei are seen. All images are to the same scale. HBD: Human β -defensin; hTERT: Human telomerase reverse transcriptase; rHagB: Recombinant hemagglutinin B. Unpublished observations from Pingel *et al.* [54]. rHagB and monoclonal antibody to rHagB were obtained courtesy of Myriam Bélanger and Ann Progulsk-Fox (Center for Molecular Microbiology and Department of Oral Biology, College of Dentistry, University of Florida, FL, USA), the Oral hTERT-immortalized human adenoid keratinocytes were obtained courtesy of Aloysius J Klingelhutz (Department of Microbiology, University of Iowa, IA, USA) and the human myeloid dendritic cells were obtained courtesy of Zoya B Kurago (Department of Oral & Maxillofacial Pathology, Radiology and Medicine, New York University College of Dentistry, NY, USA).

Figure 3. Human β -defensin 3 alters the binding of recombinant hemagglutinin B to human myeloid dendritic cells

Visualized using electron microscopy, immunogold labeling (arrows) shows that rHagB binds to the outer membrane of cells (A), which can be significantly inhibited by prior incubation with HBD3 (B). Significantly less immunogold labeling was seen on cells incubated only with HBD3 (C) and PBS (D). The number of gold particles per micrometer of dendritic cell membrane can be seen in the graph (E). * $p < 0.05$ compared with rHagB.

HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin; PBS: Phosphate-buffered saline; rHagB: Recombinant hemagglutinin B.

Unpublished observations from Dietrich *et al.* [55]. rHagB and monoclonal antibody to rHagB were obtained courtesy of Myriam Bélanger and Ann Progulsk-Fox (Center for Molecular Microbiology and Department of Oral Biology, College of Dentistry, University of Florida, FL, USA) and the human myeloid dendritic cells were obtained courtesy of Zoya B Kurago (Department of Oral & Maxillofacial Pathology, Radiology and Medicine, New York University College of Dentistry, NY, USA).

Table 1
Characteristics of human α - and β -defensins

Name	Amino acid residues (n) [*]	Gene [‡]	Net charge (theoretical pI) [§]	Predicted monoisotopic mass (Da) [§]
HNPs[¶]				
HNP1	30	<i>DEFA1</i>	+3 (8.68)	3445.56
HNP2	29	N/A [#]	+3 (8.67)	3374.52
HNP3	30	<i>DEFA3</i>	+2 (8.33)	3489.55
HNP4	34	<i>DEFA4</i>	+3 (8.70)	3827.83
HBDs^{**}				
HBD1	36	<i>DEFB1</i>	+5 (8.87)	3931.78
HBD2	41	<i>DEFB4</i>	+7 (9.30)	4331.18
HBD3	45	<i>DEFB103A</i>	+11 (10.08)	5157.71
HBD4	49	<i>DEFB104A</i>	+7 (9.45)	5855.87

^{*} The number of amino acid residues and net charge were obtained from The Antimicrobial Peptide Database [92,93,201].

[‡] Data taken from [202].

[§] The theoretical pI and the predicted monoisotopic mass were determined using the Expert Protein Analysis System (ExPASy; [203]).

[¶] In the HNPs, the first and the sixth cysteine amino acid residues, the second and fourth cysteine amino acid residues, and the third and the fifth cysteine amino acid residues are linked by disulfide bonds forming a distinct 3D structure with structurally rigid β -sheets.

[#] DEFA1 and DEFA3 encode human neutrophil peptides HNP1, HNP2 and HNP3.

^{**} In the HBDs, the first and the fifth cysteine amino acid residues, the second and fourth cysteine amino acid residues, and the third and the sixth cysteine amino acid residues are linked by disulfide bonds, also forming a distinct 3D molecule with structurally rigid β -sheets.

DEFA: Defensin- α ; DEFB: Defensin- β ; HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin; pI: Isoelectric point.

Table 2
Human neutrophil peptide α -defensins and human β -defensins in oronasal tissues, salivary glands and secretions

Oral tissues	Defensin	Comments	Ref.
<i>Oronasal tissues</i>			
Oral epithelium	HNP1, HNP2, HNP3, HBD1, HBD2, HBD3	HBD1 mRNA is strongly expressed in the broad spinous cell region and localized throughout the nucleated layers of the epithelium. HBD1 protein is present in the well-differentiated cells of the upper spinous and granular layers. HBD2 mRNA is induced mainly in the spinous and granular layers of the epithelium. HBD2 protein is present in the upper granular layers of epithelium with spotty presence in the spinous layers. HBD3 mRNA is expressed in the basal layer in healthy tissue occasionally extending into the spinous and upper layers	[94–97]
Sulcular epithelium	HNP1, HNP2, HNP3, HBD1, HBD2	HNPs are produced near neutrophils in the sulcular epithelium	[95]
Junctional epithelium	HNP1, HNP2, HNP3	HNPs are produced near neutrophils in the junctional epithelium	[95]
Connective tissue	HNP1, HNP2, HNP3	HNPs are produced near neutrophils in the connective tissue	[95]
Tongue	HBD1, HBD2, HBD3	HBD1 mRNA expression is detected in the tongue	[98–100]
Buccal mucosa	HBD1, HBD2, HBD3	There is HBD1 and HBD3 expression in the upper half of the tissue	[99,101]
Labial mucosa	HBD1, HBD2, HBD3		[99]
Mucosal floor	HBD1, HBD2, HBD3		[99]
Dental follicle	HBD1, HBD3		[99]
Dental pulp	HBD1, HBD2		[99]
Adenoid	HBD2, HBD3		[100,102]
Larynx	HNP1, HNP2, HNP3, HBD1, HBD3	Positive immunohistochemical staining of HNP1–3 in subepithelial and intraepithelial neutrophils	[100,103]
Tonsil	HBD1, HBD2, HBD3	There is strong expression of HBD2 in tonsillar tissue	[100,102]
<i>Salivary glands</i>			

Oral tissues	Defensin	Comments	Ref.
Parotid gland	HBD1, HBD2, HBD3	In the salivary glands, mRNA expression was constitutive for HBD1, frequent for HBD2 and infrequent for HBD3	[98–100,104]
Submandibular gland	HBD1, HBD2, HBD3		[99]
Small labial gland	HBD1, HBD2, HBD3		[99]
Sublingual gland	HBD1, HBD2		[99]
<i>Oronasal secretions</i>			
Saliva	HNP1, HNP2, HNP3, HBD1, HBD2, HBD3	Concentrations (µg/ml) range from 0.9 to 39.2 for HNP1, 0.1–18.2 for HNP2, 0.1–2.7 for HNP3, 0.01–00.02 for HBD1, 0.0–21.0 for HBD2 and 0.0–7052.0 for HBD3	[98,105–112]
Gingival crevicular fluids	HNP1, HNP2, HNP3, HBD1, HBD2, HBD3		[105,106,109,111,113,114]
Nasal secretions	HNP1, HBD1, HBD2		[115]

HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin.

Table 3
Human neutrophil peptide α -defensins and human β -defensins attenuate proinflammatory cytokine responses in monocytes, macrophages and dendritic cells treated with lipopolysaccharide or recombinant hemagglutinin B

Defensin	Cell	Cytokine inhibited	Ref.
HNP1	Human monocytes treated with LPS*	IL-1 β	[38]
HNP1-3	Human monocyte-derived macrophages treated with LPS [†]	IL-1 β , IL-6, IL-8, IL-10, TNF- α	[39]
HBD3	Human myeloid dendritic cells treated with rHagB [§]	IL-6, IL-10, GM-CSF, TNF- α	[40]

* LPS (20 ng/ml)

[†] LPS (1 ng/ml)

[§] rHagB from *Porphyromonas gingivalis* strain 381 (50 ng/ml).

GM-CSF: Granulocyte-macrophage colony-stimulating factor; HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin; LPS: Lipopolysaccharide; rHagB: Recombinant hemagglutinin B.

Table 4
Affinity of defensins for bacterial and viral antigens

Ligand	Analyte	Mean kinetic dissociation (K_D) constant *	Ref.
rFimA [‡]	HBD3	4.88E-07	[44]
rFimA	HNP1	4.60E-07	[44]
HIV gp120 (93TH975.15)	RC-101	4.28E-07	[51]
HIV gp120	RC-112	4.21E-07	[52]
HIV gp120 (CM235)	RC-100	2.90E-07	[51]
rFimA	HNP2	2.27E-07	[44]
HIV gp120 (CM235)	RC-101	1.93E-07	[51]
HIV gp41 (HXB2)	RC-1	6.76E-08	[53]
rHagB [§]	HBD2	5.00E-08	[44]
HIV gp120 (LAV IIIB)	RC-100	3.54E-08	[51]
HIV gp120	RC-100	3.54E-08	[52]
HIV gp120 (LAV)	RC-1	3.30E-08	[53]
HIV gp120 (LAV IIIB)	RC-101	3.12E-08	[51]
HIV gp120	RC-115	2.18E-08	[50]
HIV gp120	RC-116	1.98E-08	[50]
rHagB	HBD3	1.79E-08	[44]
rHagB	HNP1	1.08E-08	[44]
rHagB	HBD1	8.76E-09	[44]
HIV gp120 (93TH975.15)	RC-100	4.70E-09	[51]
rFimA	HBD1	3.70E-09	[44]
rHagB	HNP2	1.66E-09	[44]
rFimA	HBD2	4.08E-10	[44]

* Listed from lowest Affinity to highest Affinity.

[‡] rFimA of *Porphyromonas gingivalis* strain 381.

[§] rHagB of *P. gingivalis* strain 381.

HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin; RC: Retrocyclin; rFimA: Recombinant fimbrillin A; rHagB: Recombinant hemagglutinin B.

Table 5
Recent examples of defensins used as immunological adjuvants

Peptide	Examples	Ref.
α -defensins	HNP1, 2 and 3 induce adaptive host immunity to ovalbumin in mice. Intranasal delivery of HNPs with ovalbumin enhances ovalbumin-specific IgG, but not IgA, antibody responses	[80]
	Enhance antibody response to a tumor antigen, lymphoma Ig idiotype and also augments resistance to tumor challenge	[78]
β -defensins	HBDs induce adaptive immune responses to ovalbumin	[81]
	Defensins have potential for use in novel vaccines to combat cancer and clinically relevant diseases	[41]
	Duck defensin (Apl_AvBD2) has chemotactic and immunomodulatory activities, properties desirable for use as a molecular adjuvant with avian vaccines	[116,117]

HBD: Human β -defensin; HNP: Human neutrophil peptide α -defensin.