Mucosal Immunity and Inflammation
IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier*

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Ouellette, Andre J. Mucosal Immunity and Inflammation. IV. Paneth cell antimicrobial peptides and the biology of the mucosal barrier. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G257–G261, 1999.—The hypothesis that epithelial cells release preformed antibiotic peptides as components of mucosal innate immunity has gained experimental support in recent years. In the mammalian small intestine, Paneth cells secrete granules that are rich in α-defensins and additional antimicrobial peptides into the lumen of the crypt. The α-defensins are homologues of peptides that function as mediators of nonoxidative microbial cell killing in phagocytic leukocytes, and they are potent microbicidal agents in in vitro assays. Because certain mouse α-defensins stimulate cultured epithelial cells to secrete chloride ion, those peptides appear to be capable of interacting directly with the apical membranes of neighboring cells and perhaps influencing crypt physiology. In instances of crypt disruption or induced Paneth cell deficiency, crypt intermediate cells appear to compensate by accumulating and secreting Paneth cell antimicrobial peptides. Challenges for the future will be to understand the mechanisms of this epithelial plasticity and to show that Paneth cells contribute directly to innate immunity in the crypt microenvironment.

innate immunity; crypt epithelium; cryptdins

The epithelial monolayer that lines the mammalian small intestine is both the route of nutrient absorption and an active barrier between the external environment and the circulation. At first appearances, adaptations of the small bowel to improve nutrient uptake by amplification of absorptive surface area would seem to increase the risk of mucosal colonization by potential pathogens. Nevertheless, the bacterial load of the small bowel remains very low relative to that of the colon.

Many factors contribute to the low bacterial numbers of the small intestine. Examples include the normal physiological activities of the gut such as motility, secretion of digestive and pancreateobiliary juices, mucous secretions of goblet cells, and humoral and cytotoxic immune responses of B and T lymphocytes to specific antigens. In addition, however, epithelial cells are known to actively release gene-encoded, antibiotic peptides that contribute to a biochemical barrier against microbial colonization. In addition to the studies of small intestinal antimicrobial peptides to be reviewed briefly here, several investigators have shown that the mucosa of the airway, skin, gingiva, tongue, cornea, reproductive tract, urogenital tract, and colon also participate in innate immunity (6).

ANTIMICROBIAL PEPTIDES

The production of antimicrobial proteins and peptides is widely distributed phylogenetically. Originally described as responses of circulating hemocytes to cuticle injury in insect larvae, antimicrobial peptides are now recognized as effectors of a biochemical barrier against potential pathogens in plants, insects, amphibia, teleosts, and mammals (15). Selective pressure for the synthesis and release of antimicrobial peptides in response to infection, or the constant threat of infection, appears to have been widespread, since peptides with potent in vitro antimicrobial activities have been isolated from all phyla examined, including plants, invertebrates, and species lacking clonal immune mechanisms (14). These preformed peptide antibiotics are highly variable in primary structure, and they may be inducible, synthesized continually and accumulate in cytoplasmic granules for regulated secretion, or they may be released on a constitutive basis.

Gastrointestinal expression of antimicrobial peptides also is evolutionarily conserved. In the gastrointestinal tract of the frog, for example, magainins, the primary antimicrobial peptides of frog skin, are found in stomach glands, and they also are present in glands at the base of epithelial folds in the frog intestine (32). In invertebrates, midgut production of insect defensins becomes activated in the mosquito and Stomoxys calcitrans after consumption of a blood meal (5, 21), and the midgut of Manduca sexta larvae contains antimicrobial peptides in granules (21). These findings suggest that the production and secretion of antimicrobial peptides by mammalian intestinal epithelia is a conserved innate immune mechanism rather than a recent evolutionary development.

Most antimicrobial peptides expressed by mammalian epithelial cells are members of peptide families that mediate nonoxidative microbial cell killing by...
phagocytes (15). In polymorphonuclear leukocytes, the peptides are stored in the azurophilic granules, and they mediate killing of ingested microorganisms following phagolysosomal fusion. Epithelial peptides, on the other hand, appear to function in the extracellular compartment at the interface with the external environment (26). The colonic, airway, and reproductive epithelial cells appear to release antimicrobial peptides continually or constitutively. In the small intestine, microbicidal peptides accumulate in secretory granules of Paneth cells for apical release by regulated exocytosis. Because epithelial antimicrobial peptides are homologous to peptides in the azurophilic granules of neutrophils, e.g., defensins, and because those peptides are microbicidal in in vitro assays, they are implicated in immunity at the mucosal surface.

**ANTIMICROBIAL PEPTIDES IN PANETH CELLS**

Paneth cells are located at the base of the crypts of Lieberkühn in the small intestine of many mammalian species. They are recognized by the unusually large apical secretory granules that they release into the crypt lumen. Most small intestinal crypts are populated by Paneth cells, and, in mice, amplification of α-defensin cDNA sequences in isolated crypts by RT-PCR has shown that every crypt contains Paneth cells or Paneth cell transcripts (3). Aside from their unusual morphology, Paneth cells have certain characteristic features. For example, although they originate from the same crypt stem cells that generate all intestinal epithelial cell lineages, they differentiate during downward migration from the proliferative zone (2). In contrast, the other epithelial cell populations differentiate terminally as they ascend the crypts and migrate along the villi toward their eventual exfoliation. Also, Paneth cells have a 20 day average lifespan, unlike villous enterocytes that apopose and exfoliate into the lumen 2–5 days after emergence from the stem cell zone. Histologically, normal Paneth cells develop under germ-free conditions and from murine or human fetal intestinal xenografts in nude mice (20), demonstrating that Paneth cell ontogeny does not require luminal bacteria or dietary constituents.

Insights into Paneth cell function have been provided by analyses of their gene products. For example, secretion of lysozyme and homologues of phagocytic antimicrobial peptides implicates Paneth cells in enteric host defense. Specifically, Paneth cell lysozyme, secretory phospholipase A₂ (sPLA₂), and α-defensins have well-established antimicrobial activities when assayed in vitro (16). In mice, these gene products appear during postnatal crypt ontogeny, coincident with Paneth cell differentiation, and they are useful markers of the lineage. The secretion of this array of peptide antibiotics by the Paneth cell, the defensins in particular, is consistent with an innate immune role.

**DEFENSINS**

Two known defensin peptide subfamilies have been well described, the α- and β-defensins. Both subfamilies comprise cationic, 3- to 4-kDa peptides that contain six cysteine residues in three disulfide bonds. Although their tridisulfide arrays differ in their Cys-Cys pairings, α- and β-defensins have remarkably similar folded conformations (29). The mammalian α-defensins are major constituents of the primary granules in phagocytic leukocytes of myeloid origin, and they were one of the first antimicrobial peptide families to be recognized (6). The β-defensins occur in more tissues than the α-defensins, but they have not been detected in Paneth cells to my knowledge. They are expressed in bovine bone marrow and in the mucosa of the colon, airway, tongue, kidney, skin, and gingiva in humans and in other species. Inhibition of human β-defensin-1 antibacterial activity by the high ionic strength of airway surface fluid of cystic fibrosis patients has been implicated in microbial pathogenesis associated with the disease (4); nevertheless, the mechanisms involved remain unresolved. Several years subsequent to their discovery in neutrophils, α-defensins were identified in mouse and human Paneth cells (16).

**ENTERIC α-DEFENSINS**

α-Defensins are abundant constituents of mouse and human Paneth cell granules. Human Paneth cells code for two α-defensin peptides, HD-5 and HD-6, and mice express numerous Paneth cell α-defensin isoforms, termed “cryptdins” for crypt defense. Six cryptdins have been purified to homogeneity (16). Immunohistochemical studies of small intestinal sections have shown that antibodies to mouse cryptdin-1 and recombinant HD-5 react exclusively with Paneth cells of the respective species (17, 25). Immunogold detection experiments show that the antigen is distributed uniformly in Paneth cell granules (17). In mice, individual isoforms differ in relative abundance in that whole organ recoveries of cryptdins-1, -2, -5, and -6 are equivalent but levels of cryptdins-3 and -4 are much lower. The primary structures of cryptdins-4 and -5 diverge markedly from each other and from the majority of cryptdins that are variably substituted variants of cryptdin-1 (16). Cryptdins-2 and -3, for example, differ in sequence only at position 10 (Thr vs. Lys, respectively). Cryptdin-4 is the most cathodal enteric defensin and the first α-defensin to contain a chain-length variation between the fourth and fifth cysteine residues. Furthermore, cryptdin-4 gene is unique because it is expressed differentially along the length of the small bowel, since cryptdin-4 mRNA and peptide are not detected in duodenum but occur at highest levels in distal ileum. The mechanisms regulating this pattern of expression are not known.

**α-DEFENSIN BIOSYNTHESIS**

Most of the defensin peptide in mature phagocytic cells appears to be fully processed via a pathway that involves two primary cleavage steps (7). The human α-defensin precursors are cleaved sequentially over a 4- to 24-h period to yield intermediates of 75 and 56 amino acids, and agents that neutralize the acidic
subcellular compartment diminish the conversion rate. The anionic charge of many α-defensin precursor prosegments has been suggested to have a role in neutralizing the basic charge of the functional peptide. Also, addition of the propeptide to in vitro antimicrobial peptide assays inhibits neutrophil α-defensin activity, suggesting that the prodomain may be cytoprotective. Deletional mutations in the COOH-terminal region of the HNP-1 human neutrophil α-defensin prosegment impair pro-HNP-1 processing and targeting to granules, suggesting that residues proximal to the cleavage site may be involved in the recognition and cleavage steps. Matrilysin (MMP-7), a metalloproteinase expressed abundantly by Paneth cells of mouse small bowel (30), has been identified as the enzyme that processes and activates mouse cryptdin precursors posttranslationally (C. L. Wilson, A. J. Ouellette, D. P. Satchell, T. Ayabe, Y. S. López-Boado, J. L. Stratman, S. J. Hultgren, L. M. Matrisian, and W. C. Parks, unpublished observation). This finding is consistent with the processing and activation of members of the large and diverse procatheclicidin antimicrobial peptide family by neutrophil elastase (24).

Paneth cell α-defensins are coded by highly conserved genes that consist of two exons separated by an intron of ~500 bp, and transcripts of these genes are ~1 kb in length. The 5′ untranslated region, signal peptide, and prosequence are coded by exon 1, and exon 2 encodes the α-defensin peptide and the 3′ untranslated region. Paneth cell α-defensin genes map in proximity to the myeloid α-defensin and β-defensin genes at 8p21–8pter in humans and at an homologous locus on proximal chromosome 8 in mice. Levels of α-defensin mRNA and peptide appear to be approximately the same in human and mouse Paneth cells, but a series of gene duplication events expanded the gene family in mice, producing over 20 different isoforms. Genetic evidence shows that 17 of these isoforms are expressed in a single crypt from mouse jejunum. However, intestinal levels of deduced cryptdin-7 to cryptdin-19 are judged to be much lower than those of cryptdins 1–6, based on the low relative cloning frequency of these cDNAs and the fact that cryptdin-7 to cryptdin-19 have not been recovered as abundant peptide components of intestinal extracts.

Peptides recognized originally as Paneth cell α-defensins also are expressed by nonintestinal epithelia. For example, HD-5 transcripts and peptides have been found in human female reproductive tract epithelium (19, 27). Cryptdins are present in Leydig cells and Sertoli cells of mouse testis (9), including an α-defensin mRNA that has not been detected at the cDNA or peptide level in mouse gut (Ouellette, unpublished observation). Similarly, α-defensins isolated from rabbit kidney bear little resemblance to the rabbit myeloid α-defensins, suggesting that the renal epithelium, a well-established site of expression and release of β-defensins (4), may express α-defensins as well (1, 31). Collectively, these findings suggest that α-defensins are potential mediators of innate immunity on numerous mucosal surfaces.

**ANTIMICROBIAL ACTIVITIES OF PANETH CELL α-DEFENSINS**

Mouse and human Paneth cell α-defensins are potent antimicrobial agents with selective activities against several varied microbial cell targets. A recombinant form of HD-5 is active against several species of bacteria as well as C. albicans, and the peptide remains active even after partial proteolysis, an indication of its potential to function within the environment of the intestinal lumen (18). In vitro assays of cryptdin antibacterial activities showed that each of the six peptides was microbicidal, except for cryptdin-2 (16). In suspension, E. coli ML35 cells were killed rapidly by cryptdins-1 and cryptdins-3 to -6, and, in each case, <1% survival was observed after 15 min of incubation. Because cryptdins-2 and -3 differ only at amino acid position 10 (Thr vs. Lys, respectively), that residue position appears to modulate killing activity in the context of those particular primary structures. Trophozoites of Giardia lamblia are highly sensitive to cryptdins-2 and -3, but cryptdins-1 and -6 have little effect on survival of this enteric protozoal pathogen as determined by trypan blue exclusion (16). Comparing the primary structures of these peptides implicates amino acid residue 15 in this activity because giardicidal cryptdins-2 and -3 contain arginine at position 15 but inactive cryptdins-1 and -6 contain a glycine at residue 15. By analogy with the crystal structure of neutrophil defensin HNP-3, amino acid 15 is predicted to be on the peptide surface in a conserved turn and possibly important in interactions with eukaryotic cell envelopes (16).

The mechanisms of Paneth cell defensin antimicrobial activity are not known, but, by analogy with neutrophilic α-defensin homologues, it is likely that they permeabilize the target cell envelope leading to dissipation of electrochemical gradients. The human and rabbit neutrophil α-defensins achieve cell killing by membrane disruption, but, as peptide-to-membrane interaction studies suggest, the details of the killing mechanism may be very different from peptide to peptide. Despite their conserved tridisulfide structure, amphipathicity, and β-sheet backbones, human neutrophil α-defensin HNP-2 is a noncovalent dimer, but NP-1, an α-defensin from rabbit neutrophils, is a monomer (29). Functionally, the dimeric HNP-2 peptide forms large, ~20 Å, stable multimeric pores after insertion into model membranes; in contrast, NP-1 generates short-lived defects in phospholipid bilayers. The interactions of six rabbit neutrophil α-defensins with large unilamellar vesicles of defined lipid composition showed that the membrane phospholipids, cardiolipin in particular, strongly affect their permeability to individual antimicrobial peptides (10). Given these considerations, it seems prudent to avoid predicting mechanisms for microbial cell killing by Paneth cell α-defensins.

**BIOLOGY OF PANETH CELLS AND α-DEFENSINS IN THE CRYPT**

Paneth cell secretion can be stimulated by cholinergic agonists that appear to act by activation of G...
proteins coupled to muscarinic receptors (22). Pilocarpine, bethanachol, and the nonspecific G protein activators NaF and AlCl3 induce massive Paneth cell degranulation (23), and muscarinic antagonists inhibit Paneth cell degranulation. In vitro, carbamylcholine interacts directly with isolated mouse ileal crypts, specifically mobilizing cytosolic intracellular calcium in Paneth cells and not affecting calcium flux in the other epithelial cell populations of the crypt. The coupling of specific G proteins to Paneth cell muscarinic receptors remains to be demonstrated, but these findings are consistent with their involvement in the regulation of Paneth cell secretion.

Physiological salt and water secretion from the intestine depends on the activation of chloride channels in the apical membrane of epithelial cells lining the crypt, the secretory gland of the intestine. Evidence suggests that mouse Paneth cell α-defensins may participate in that process, since cryptdins-2 and -3 activate chloride channels when applied apically to monolayers of human T84 intestinal epithelial cells (13). The chloride secretory effect is reversible, time and dose dependent, and isoform specific, since cryptdins-2 and -3 cannot form the channel but cryptdins-1 and cryptdins-4 to -6 can. In vitro, modulation of intestinal and renal cell volume, alteration of epithelial monolayer barrier integrity, and the fact that certain peptides may be cytotoxic to mammalian cells suggest that Paneth cell α-defensins may be multifunctional.

PANETH CELL DYNAMICS IN THE CRYPT

Conditions that disrupt normal crypt cell biology appear to recruit new cells to activate Paneth cell α-defensin genes and accumulate the corresponding peptides. For example, in mice that express attenuated diphtheria toxin A fragment or SV40 large T antigen transgenes under the control of a functional mouse cryptdin-2 gene promoter, the crypts undergo a transient Paneth cell deficiency (8). Up to 8 wk of age, small intestinal crypts of transgenic mice lack apparent Paneth cells, and the crypts are occupied by undifferentiated crypt columnar cells. During this period of Paneth cell deficiency, numbers of intermediate cells increase, and those cells accumulate electron-dense, secretory granules that contain elevated levels of cryptdin(s) and SPLA2 (8). Thus Paneth cell deficiency appears to induce a compensatory response in crypt intermediate cells, altering their genetic repertoire to produce and secrete Paneth cell antimicrobial peptides. These mice may be useful for determining the levels of α-defensin expressed by these activated intermediate cells, for learning whether defensins or newly activated antimicrobial peptide gene products are made during Paneth cell deficiency, and for characterizing the responses of the transgenics to oral challenge with microbial pathogens.

Recent findings suggest that the small intestinal crypt epithelium may communicate with and respond to T lymphocytes. For example, infection of mice with Trichinella spiralis induces intestinal goblet cell hyperplasia that appears to be mediated by T helper cells (11). During the course of infection with this parasite, the number of Paneth cells and goblet cells in crypts increases (M. Kamal, D. Wakelin, and Y. R. Mahida, personal communication). Furthermore, Z. Alnadjim and T. A. Barrett of Northwestern University Medical School have shown that T cell activation induced by rapid and dramatic crypt cell apoptosis followed by an approximately threefold increase in the number of crypt cells positive for eosinophilic granules (personal communication). It should be of interest to define the soluble mediators and the signaling pathways that induce the crypt epithelium to modify its genetic programs.

Collectively, the experiments in Paneth cell-deficient transgenic mice, in Trichinella infection, and in the experimental T cell activation models suggest that the crypt epithelium is capable of some plasticity with respect to the repertoire of genes that crypt cell populations may express. Part of that response includes a compensatory increase in production of secretory products, including antimicrobial peptides that normally are expressed at significant levels only by Paneth cells. Studies to identify the mechanisms of that apparent plasticity, to determine its relation to innate immunity in the crypt microenvironment, and to perhaps employ these gene products as markers of immunopathogenesis of gastrointestinal diseases may prove to be informative.

I thank Drs. Terrence A. Barrett, Charles L. Bevins, Wayne Lencer, Yashwant R. Mahida, Michael E. Selsted, and Carole L. Wilson for useful discussions and for permission to cite their unpublished findings.

This work was supported by National Institutes of Health Grants DK-44632, DK-33506, and HD-31852.

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