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Defensins and Other Antimicrobial Peptides and Proteins

Chapter 6

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INTRODUCTION

Endogenous antimicrobial peptides are widely distributed among vertebrates. Most are amphiphilic, polycationic molecules with an α-helical, cystine-stabilized β-sheet, or proline-rich structure. They represent elements of a robust, ancestral animal immune system that predates the advent of lymphocytes and immunoglobulins. Secreted and cell-associated antimicrobial peptides enable their hosts to resist incursions by potential pathogens. From a pathogen's perspective, these peptides present a series of barriers to evade or overcome. In humans (and other mammals), defensins and cathelicidins are the principal antimicrobial peptides of neutrophils and epithelial cells. Many mucosal surfaces are bathed by antimicrobial proteins, including lysozyme, lactoferrin, secretory leukoprotease inhibitor (SLPI), and secretory phospholipase A2.

Defensins

All defensins have a largely β-sheet structure and contain three intramolecular cystine disulfide bonds. The smallest, θ-defensins, are circular peptides that contain 18 residues, α-defensins have between 29 and 35 residues, and β-defensins have up to 45 **(Fig. 6.1)**. To date, more than 100 α-defensin, β-defensin, and θ-defensin molecules have been reported, and humans express six different α-defensins and *at least* four β-defensins. Additional human defensin/defensinlike genes exist in five different chromosomal loci, but their expression and properties remain to be characterized. α-Defensins occur in the neutrophils of humans, rats, rabbits, guinea pigs, and hamsters, but not in those of mice, horses, pigs, and sheep. Although the small intestinal Paneth cells of mice express at least 6 (Ouellette *et al.* 2000; Selsted *et al.* 1992) and possibly as many as 20 α-defensins (Ouellette and Selsted 1996), only two very dissimilar defensin species are found in human Paneth cells (Mallow *et al.* 1996). Two atypical α-defensins exist in rabbit kidney, but their functions remain uncertain (Bateman *et al.* 1996; Wu *et al.* 1998). Thus far, with the exception of rabbit alveolar macrophages, mononuclear phagocytes are not known to produce appreciable amounts of α-defensins.

Although many human epithelial cells constitutively express human β-defensins *(HBD-1)*, this peptide is particularly abundant in the distal renal tubules and in other parts of the genitourinary tract. *HBD-2*, originally recovered from psoriatic skin (Harder *et al.* 1997), is induced in various epithelia by inflammatory signals. In the epidermis, it is present in very low amounts unless induced by interleukin-1 (IL-1) (Liu *et al.* 2003). The *HBD-3* gene was found by genomic searching (Jia *et al.* 2001) and the corresponding peptide was isolated from psoriatic skin (Harder *et al.* 2001). *HBD-4* is highly expressed in the testis (epididymis) and less abundantly in the gastric antrum (Garcia *et al.* 2001b). Since about 50 different β-defensin genes have been identified in the mouse (Scheetz *et al.* 2002), sorting out their functions and relationships to human biology is unlikely to be a simple task.

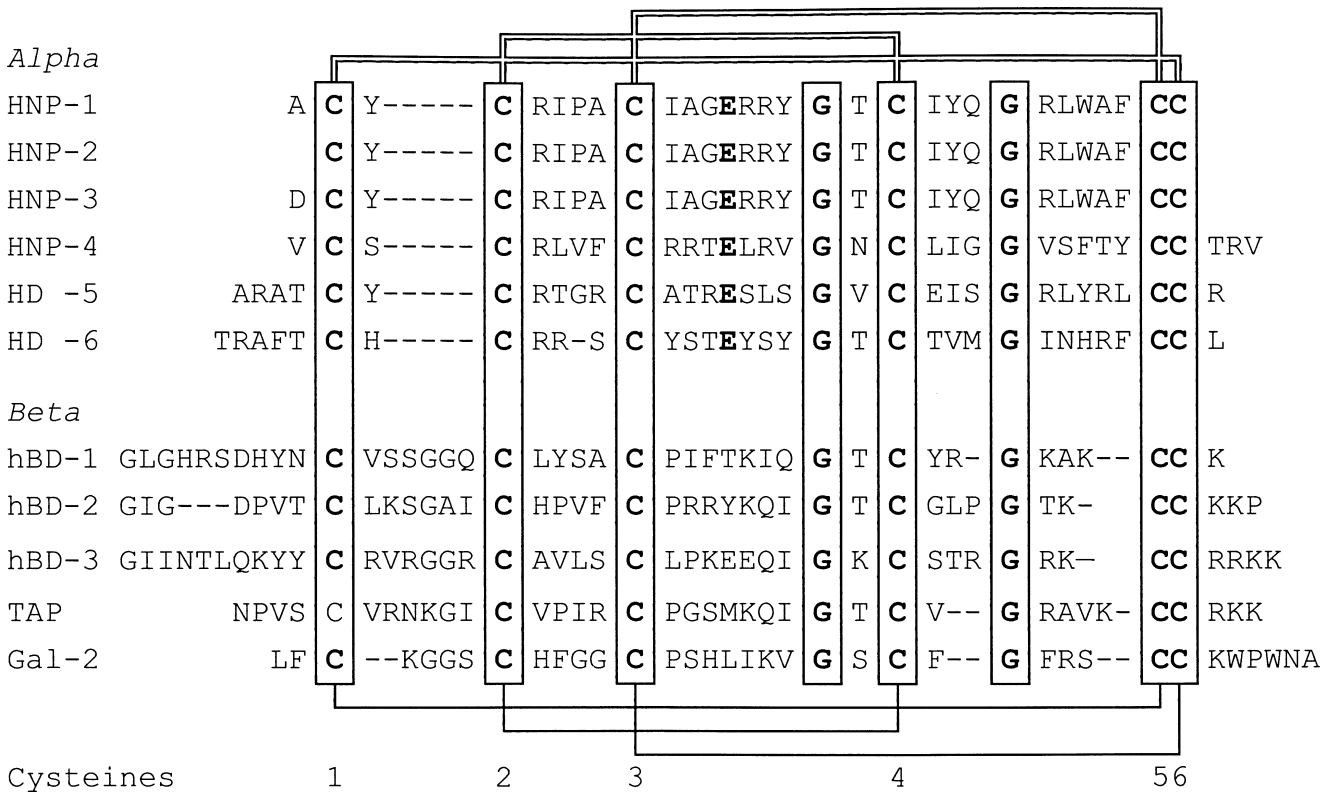

Fig. 6.1. Primary structures of representative α-defensins and β-defensins. The α-defensins (and the cells that contain them) include HNP 1–4 (human neutrophils), HD 5 and 6 (human Paneth cells), RabNP-1 (rabbit granulocytes and alveolar macrophages), and RatNP-1 (rat neutrophils). The β-defensins (and the cells that contain them) include Gal-2 (gallinacin-2, chicken granulocytes), TAP (tracheal antimicrobial peptide, bovine respiratory tract epithelia), BNBD-12 (bovine granulocytes), and hBD-1, -2, and -3 (human epithelial cells). The generally conserved or invariant residues found in both subfamilies have been boxed. The respective cysteine connectivity of α-defensins and β-defensins is shown above and below the sequences.

GENERAL PROPERTIES OF DEFENSINS

Structure of mature defensins

Figure 6.1 shows the primary amino acid sequences of eleven defensins. The six α-defensins are of human origin: HNP 1–4 from leukocytes, and HD-5 and 6 from intestinal cells. The five α-defensins include human hBDs 1–3, bovine "tracheal antimicrobial peptide" (TAP), and gallinacin-2 from chicken leukocytes. Highly conserved residues are boxed and/or bolded. Cysteines 5 and 6 are contiguous and near the carboxy-terminus in both α-defensins and β-defensins, and one of them is covalently linked to the cysteine nearest the amino terminus. Consequently, defensins are complexly folded, macrocyclic molecules.

By x-ray crystallography, human defensin HNP-3 is an elongated ellipsoid, 26 × 15 × 15 Å, dominated by a three-stranded, cystine-stabilized antiparallel β-sheet (Hill *et al.* 1991). The highly conserved Arg^{6} and Glu^{14} residues form a salt bridge spanning the only non–β-sheet portion of the molecule and the invariant $glycine^{24}$ occupies the third position of a Type I turn. HNP-3 crystallizes as a dimer that configures a six-stranded β-sheet stabilized by hydrophobic interactions and hydrogen bonds. That the invariant $glycine^{18}$ is located at the dimer interface suggests that dimer formation may be an essential element of defensin-mediated activity. A dimer of the human α-defensin HNP-3 is illustrated in **Figure 6.2**.

Crystallographic analyses of *HBD-1* (Hoover *et al.* 2001) and *HBD-2* (Hoover *et al.* 2000) found very similar structures for the monomers, including the presence of a short, N-terminal, α-helical segment not present in α-defensins. It is noteworthy that a similarly positioned helical region exists in the defensinlike antimicrobial peptides of insects and plants (*e.g.*, wheat purothionine). The solution structures reported for HBDs 1, 2, and 3 show them to have similar tertiary structures, with a short helical segment preceding a three-stranded antiparallel beta-sheet (Schibli *et al.* 2002). Whereas *HBD-1* and *HBD-2* were monomeric in solution, *HBD-3* was a dimer.

Structural studies on *HBD-2* also revealed some structural similarities with MIP-3α/CCL20, a chemokine showing high affinity for CCR-6 (Perez-Canadillas *et al.* 2001). Both molecules have a rigid conformation of the N-terminal CC motif, as well as the narrow groove between the N-loop and the β2-β3 hairpin. Interestingly, *HBD-2* also binds to CCR-6 and exhibits chemoattractant activity for dendritic cells

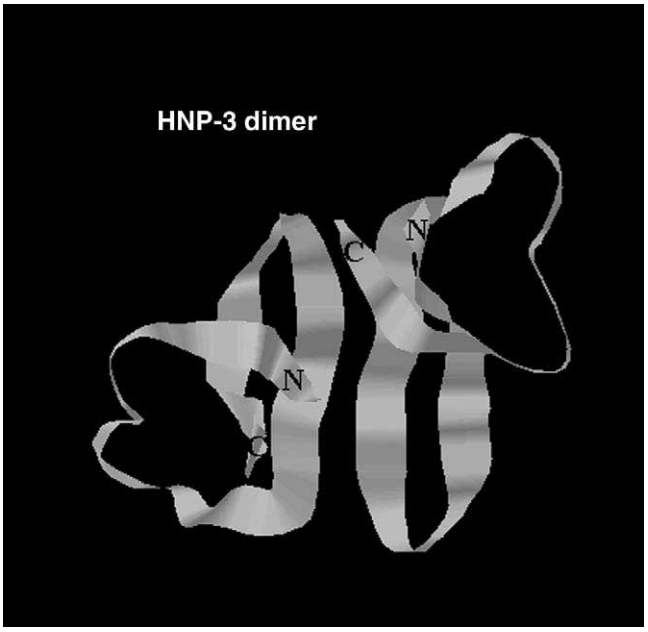

Fig. 6.2. A dimer of human α-defensin HNP-3. The figure is based on coordinates obtained from X-ray crystallography (Hill *et al.* 1991).

through its interaction with this chemokine receptor (Yang *et al.* 1999).

The θ-defensins, initially discovered in rhesus macaque neutrophils, have a remarkable structure, so far unique among mammalian peptides. These 18–amino acid (aa) peptides with three intramolecular disulfide bonds have a cyclic amide backbone (Tang *et al.* 1999; Leonova *et al.* 2001), formed from two nonapeptides by a novel posttranslational ligation reaction. The nonapeptides are encoded by two θ-defensin genes very similar to conventional α-defensins except that they are shortened by a "premature" stop codon, and contain only three cysteines. Either homodimers or heterodimers can be joined to form a mature θ-defensin peptide.

Structure and location of defensin genes

Many different defensin genes have been sequenced. The myeloid α-defensin (DEFA) genes have similar layouts, with three exons that correspond approximately to the 5′-untranslated region, signal sequence/propiece, and mature defensin region (Linzmeier *et al.* 1993). The genes for HD-5 and 6, α-defensins expressed predominantly within the human intestine, have only two exons as do the DEFB genes that encode β-defensins, suggesting that the evolution of α-defensins involved both deletional and insertional events. Although dissimilar in their genomic sequence, the genes for HD-5 and HD-6 show considerable sequence similarity in their proximal 5′-flanking regions, which presumably contain elements involved in their tissue-specific expression (Mallow *et al.* 1996; Salzman *et al.* 2003). The DEFA genes encoding human myeloid and enteric α-defensins are clustered on human chromosome 8 (Linzmeier *et al.* 1999). The human DEFB genes that encode *HBD-1*, HDB-2, and HDB-3 contain two exons and are all within a few hundred kilobases from the myeloid α-defensin locus on chromosome 8p23 (Liu *et al.* 1997; Peng *et al.* 2001). This region is also home to a DEFT pseudogene that is highly homologous to the genes encoding the α-defensins of rhesus monkeys. θ-defensin (DEFT) genes evidently arose in an Old World monkey when the mutation of a preexisting α-defensin gene placed a premature stop codon within Exon 3 of a DEFA gene. Additional defensin and defensinlike genes have been identified in four other chromosomal loci, but their tissue expression and biology remain to be characterized (Schutte *et al.* 2002).

Activities of defensins

Gram-positive and gram-negative bacteria

A review of the antimicrobial activity of defensins against gram-positive and gram-negative bacteria is available (Lehrer *et al.* 1993). Briefly, defensins are effective broad-spectrum microbicides, especially when present in high local concentrations or acting in low–ionic strength media. High local concentrations occur within phagolysosomes, in the capillary-like lumen of small intestinal crypts, and at interfaces between effector and target cells.

Rabbit defensins NP-1 and NP-2 bind with high affinity to *Pseudomonas aeruginosa* PAO1, forming small surface blebs and permeabilizing its outer membrane. This process, sometimes called "self-promoted uptake" (Sawyer *et al.* 1988), allows the polycationic peptide molecules to displace divalent cations that link adjacent lipopolysaccharide (LPS) molecules. The consequent permeability changes allow otherwise excluded molecules, such as lysozyme, to enter the periplasmic space and attack the bacterium further. Viljanen and coworkers (Viljanen *et al.* 1988) noted that human defensins increased the outer membrane's permeability to hydrophobic probes (e.g., rifampin) in *P. aeruginosa*, *Escherichia coli,* and *Salmonella typhimurium*. Bactericidal concentrations of HNP-1 permeabilized the outer and inner membranes of *E. coli*, causing immediate and simultaneous cessation of macromolecular synthesis and respiration (Lehrer *et al.* 1989a).

Permeabilization of microbial cell membranes is a hallmark of defensin-mediated antimicrobial activity. Especially with human defensins, such permeabilization is prevented by treatments that inhibit the target cell's metabolism, growth, or transmembrane protonmotive force (Lehrer *et al.* 1989a). Defensins permeabilize artificial phospholipid membranes only when a transmembrane electronmotive force of sufficient magnitude and correct polarity is applied (Kagan *et al.* 1990). Membrane conductance increases as the second to fourth power of the defensin concentration, suggesting that two to four defensin molecules interact to form a channel in this system. Wimley and associates (Wimley *et al.* 1994) examined interactions between human defensins and lipid bilayers. They found that HNP-2 (net charge, +3) bound to and permeabilized unilamellar vesicles composed of anionic phospholipids, but not those formed of electroneutral phospholipids. Data from experiments with entrapped markers of

different sizes suggested that multimeric HNP-2 formed aqueous pores with a maximum diameter of ~25 Å. If such pores formed in bacterial membranes, they should allow passage of defensin monomers and even dimers (~26 × 15 × 15 Å) to more interior sites.

Certain bacteria are intrinsically resistant to defensins, including *Neisseria gonorrhoeae* (Qu *et al.* 1996a), *Burkholderia cepacia, Burkholderia pseudomallei* (Jones *et al.* 1996), and *Brucella spp*. (Martinez de Tejada *et al.* 1995). What can make bacteria resistant has been partially explained by identifying mutations that *increase* bacterial susceptibility to antimicrobial peptides. Studies on the intracellular enteric pathogen *S. typhimurium* revealed the importance of the two-component regulatory system, PhoP/PhoQ. PhoQ is a magnesium-binding sensor kinase that responds to ionic and other environmental changes by phosphorylating PhoP, a DNA-binding protein that can activate numerous genes, including some needed for resistance to antimicrobial peptides and intracellular survival (Miller *et al.* 1990; Chamnongpol *et al.* 2003). These include important aspects of LPS structure (Ernst *et al.* 2001), and expression of an outer membrane protease that cleaves, and presumably inactivates, certain antimicrobial peptides (Guina *et al.* 2000).

Studies with *Staphylococcus aureus* associated its relative resistance to defensins and other cationic antimicrobial peptides to the modification of its membrane phospholipids with L-lysine (Kristian *et al.* 2003) and of its lipoteichoic acids with D-alanine (Collins *et al.* 2002). Both modifications reduce electrostatic binding of the peptides to the staphylococcal surface. *N. gonorrhoeae* may owe its defensin-resistance to an energy-dependent "mtr" efflux pump (Shafer *et al.* 1998). These findings suggest that interactions with cationic antimicrobial peptides may have profoundly influenced bacterial evolution.

In summary, the effect of defensins on microbes can be envisioned to occur in stages. Electrostatic adsorption to anionic sites on or near the target cell's membrane or (for certain defensins) to sugars in microbial glycoproteins or LPS result in locally high concentrations that promote defensin aggregation and multimer formation. Insertion of defensins into microbial membranes is assisted by their positive charge and by the target cell's transmembrane potential. The ensuing membrane disruption and channel formation permit intracytoplasmic entry of defensins and allow essential microbial components to leak out. Unless repaired, these events lead to irreversible target cell injury.

Fungi

Mammalian defensins can kill various fungal pathogens, including *Candida* spp., *Cryptococcus neoformans*, and hyphae and germinating spores of *Rhizopus oryzae* and *Aspergillus fumigatus* (reviewed in Lehrer *et al.* 1993). In recent years, the antifungal properties of defensins have been studied more by botanists and agriculturists than by immunologists. This reflects the importance of fungi as plant pathogens (Thomma *et al.* 2002) and the likely utility of transgenic plants. Plant defensins, like those of mammalian origin, probably owe their antifungal properties to an ability to induce membrane permeabilization (Thevissen *et al.* 1999).

Mycobacteria, spirochetes, and protozoa

Both human and rabbit defensins show activity against mycobacteria, including *M. tuberculosis* (Miyakawa *et al.* 1996; Sharma *et al.* 2000) and *M. avium-intracellulare* (Ogata *et al.* 1992), even in the presence of divalent cations or physiologic salt concentrations. Although the plasma membrane of *M. tuberculosis* is the principal target for HNP-1 action, its DNA may be a secondary target (Sharma and Khuller 2001). Human neutrophils can ingest and kill both *M. avium* (Hartmann *et al.* 2001) and *M. tuberculosis*, and the latter is apparently subdued by nonoxidative mechanisms (Kisich *et al.* 2002). *In vivo,* mycobacteria that evaded or survived the attentions of neutrophils would likely find a defensin-free sanctuary once resident within a macrophage, because the only mammalian macrophages known to contain significant quantities of defensins are alveolar macrophages of the rabbit.

In a rabbit model of syphilis, large local amounts of defensins were seen during the first 24 hours. This subsided, but reappeared on days 10 to 16, when healing began (Borenstein *et al.* 1991). The Nichols strain of *Treponema pallidum* was neutralized by rabbit defensins *in vitro* and *in vivo. Borrelia burgdorferi*, the causative agent of Lyme disease, is also susceptible to defensins (Lusitani *et al.* 2002).

The effect of defensins on protozoans has received little study, at least in vertebrates. Human α-defensin HNP-1, mouse Paneth cell defensin-2 and defensin-3 (cryptdins), and rabbit α-Defensin NP-2 killed trophozoites of *Giardia lamblia*, especially when the concentrations of sodium chloride and divalent cations were low (Aley *et al.* 1994).

Antiviral properties

Both α-defensins (Zhang *et al.* 2002) and θ-defensins (Cole *et al.* 2002) can protect cells from infection by HIV-1 *in vitro.* Persons infected with human immunodeficiency virus–1 (HIV-1) whose α,β CD8 T cells maintain the ability to produce and release α-defensins may exhibit relative resistance to developing acquired immune deficiency syndrome–defining (AIDS-defining) symptoms, compared with HIV-infected subjects whose T cells are defective in this regard (Zhang *et al.* 2002). θ-Defensins act by blocking an early step in the uptake process, and their ability to do so correlates with their ability to bind gp120 with high affinity (Cole *et al.* 2002). α-defensins also bind gp120 with high affinity, but their mechanisms of action against HIV-1 has not yet been defined.

α-Defensins also protect cells from infection by herpes simplex virus (HSV) types 1 and 2 in tissue culture media (Daher *et al.* 1986), by interfering with an early step in the infection process (Sinha *et al.* 2003). Human and rabbit α-defensins also neutralized vesicular stomatitis and influenza A/WSN virus, but lacked significant activity against cytomegalovirus, reovirus, and echovirus (Daher *et al.* 1986). Among the nonenveloped viruses, only adenoviruses

have so far been reported to be susceptible to defensins (Gropp *et al.* 1999; Bastian and Schafer 2001).

Cytotoxic activity

Purified human defensins kill various normal and tumor cell targets in a concentration and time-dependent fashion (reviewed in Lehrer *et al.* 1993) and show synergistic activity when combined with sublytic concentrations of hydrogen peroxide. Defensin molecules bound to mammalian target cells with biphasic kinetics, similar to those with *Candida albicans*. K562 cells exposed to 20 μg/ml of radiolabeled HNP-1 bound approximately 4.3×10^9 defensin molecules per cell. This initial binding did not cause cytolysis and was comparable in defensin-resistant and defensin-sensitive target cells. After 5 to 10 minutes, sensitive targets became permeable to trypan blue (molecular weight = 960 Da) and manifested enhanced transmembrane ion flux. Membrane-bound defensin molecules became progressively more difficult to dislodge by adding serum, and after 3 to 4 hours, the cells began releasing ^{51}Cr-labeled cytoplasmic components. Strand breaks and adenosine triphosphate–ribosylation (ADP-ribosylation) were first detected in K562 and Raji targets 6–8 hours after incubation with HNP, and these increased to maximal levels by 18 hours. DNA was not degraded into nucleosome-sized fragments. Other experiments suggested that the initial defensin-induced pores were voltage dependent and that defensin-mediated injury to mammalian cells depended on an energized target cell membrane. Since defensins are bound by α2-macroglobulin and other normal serum components, it is not surprising that defensins were minimally, if at all, cytotoxic in the presence of serum. Whether and where defensins are cytotoxic *in vivo* is not known.

Other properties of defensins

Although most studies of defensins have examined their antimicrobial properties, considerable evidence suggests that some defensins also play other roles, including immunomodulation, hormonal regulation, opsonization, and stimulating wound repair. Human neutrophil defensins are chemotactic for human monocytes (Territo *et al.* 1989), T-cells (Chertov *et al.* 1996), and immature dendritic cells (Yang *et al.* 2000) *in vitro*, so that their release from neutrophils could provide a signal to mobilize immunocompetent mononuclear cells. Defensins also induce the synthesis of interleukin-8 (IL-8), a C-X-C cytokine, by human airway epithelial cells, possibly providing a mechanism to recruit additional neutrophils to sites of inflammation (Van Wetering *et al.* 1997). The α-defensins of human neutrophils were shown to act as adjuvants to enhance systemic IgG antibody response (Lillard *et al.* 1999). Murine β-defensin MBD-2 acted as an adjuvant for tumor immunization, by providing a costimulatory signal via the toll-like receptor 4 (Biragyn *et al.* 2002). It is not yet clear whether these activities are shared by other defensins.

Rabbit NP-3a ("corticostatin") and certain other α-defensins bound reversibly to the adrenocorticotropic hormone (ACTH) receptor of rat adrenal cells *in vitro* and inhibited ACTH-stimulated steroidogenesis (Zhu and Solomon 1992). Rabbit defensins NP-1 and NP-2 greatly enhanced the ability of rabbit alveolar macrophages to ingest bacteria and fungi under serum-free conditions (Fleischmann *et al.* 1985). Defensins were mitogenic for fibroblasts (Murphy *et al.* 1993), and small daily injections of rabbit defensins accelerated wound healing in rats (Kudriashov *et al.* 1990). Guinea pig defensins were reported to release histamine from rat mast cells *in vitro* (Yamashita and Saito 1989).

DEFENSINS IN MYELOID CELLS

Role in mucosal host defense

Most normal mucosal surfaces contain considerably more epithelial cells than neutrophils or macrophages. Notable exceptions include the gingival crevices that surround teeth, which are bathed in high concentrations of neutrophils and their components. Neutrophil defensin concentrations as high as 1 mg/ml have been detected in such fluids (T. Ganz and K. Miyasaki, unpublished) and could play an important role in controlling local microbial proliferation.

In other locations, neutrophils and macrophages are rapidly recruited to epithelial surfaces when the primary mucosal defenses are overwhelmed by microbial invaders. The important function of these secondary defenses is illustrated by the particularly severe impact of neutropenia on the integrity of oral and gastrointestinal mucosae and the characteristic involvement of periodontal tissues in congenital or acquired neutrophil disorders.

Cellular localization of myeloid defensins

Each human neutrophil contains several thousand cytoplasmic granules that are divided into three principal subtypes, called primary (or azurophil), secondary (or specific), and tertiary. These subtypes can be distinguished by buoyant density, when they are made during the neutrophil's maturation, their response to secretagogues, and their composition. About 30% to 50% of the protein content of the azurophil granules consists of defensins. Relatively small amounts of defensins are released extracellularly after phagocytosis or secretagogue exposure, as the peptides are preferentially delivered phagosomes that contain opsonized bacteria (Joiner *et al.* 1989). This targeted delivery of defensins not only places them where they are most needed, but it also helps minimize any potential cytotoxicity to host tissues. The high concentrations of defensins in human neutrophils are not unique: α-defensins are similarly plentiful in the granules of rabbit neutrophils (~10–15 μg/million cells) and rabbit alveolar macrophages (~2 μg/million cells). Although myeloid defensins (HNPs) 1–4 are most abundant in neutrophils, HNP-1 is also highly expressed by natural killer (NK) cells (Obata-Onai *et al.* 2002; Agerberth *et al.* 2000) and can be produced by other lymphocytes (Agerberth *et al.* 2000).

Structure and production of myeloid defensin precursors

Although human blood neutrophils contain about 5 μg of defensins per million cells, defensin mRNA is not detectable in Northern blots of mature neutrophils, indicating that defensin synthesis is restricted to the neutrophil's bone marrow precursors. Myeloid α-defensins are synthesized as 90–95-aa prepropeptides with a generally well-conserved signal sequence, followed by an anionic propiece and the C-terminally placed defensin domain. Nevertheless, nearly all of the defensin in neutrophil azurophil granules is found to be completely processed to 29–30-aa mature peptides (Harwig *et al.* 1992).

Posttranslational defensin processing has been studied in metabolically labeled HL-60 and CML cells (Valore and Ganz 1992). Their earliest defensin intermediate contained 75 aa and arose by cotranslational removal of the 19-residue signal sequence. Considerable quantities of this 75-aa form were secreted into the medium, and the remainder was proteolytically processed over 20 hours via a 56-aa intermediate into the mature 29-aa and 30-aa defensins. Mature human neutrophils also contained minor amounts (0.25% of total defensins) of incompletely processed 39-aa, 34-aa, and 32-aa defensins (Harwig and Ganz 1992).

This pattern of synthesis and posttranslational processing was substantially reproduced when the HNP-1 defensin cDNA was transduced into defensinless murine 32D and 32D cl3 cells (Liu and Ganz 1995). These transgenic lines accumulated mature defensin in acidified vacuoles corresponding to lysosomes or immature granules. In contrast, two transgenic, defensin-producing nonmyeloid cell lines (an embryonic NIH 3T3 cell line and the pituitary adenoma cell line AtT-20) failed to process the 75-aa prodefensin, indicating that the requisite processing pathway may be tissue specific.

To study the role of the propiece in preproHNP-1, a series of in-frame deletions between the signal peptidase site and the aminoterminus of the mature defensin region (aa 21–64) was constructed, packaged, and transduced into the 32D cl3 granulocytic cell line. Deletions in the amino-terminal two-fifths of the propiece had only minor effects on defensin biosynthesis and did not interfere with accumulation of mature defensin in the granules of 32D cl3 cells. Deletions in the carboxyterminal three-fifths of the propiece diminished net defensin synthesis, blocked constitutive secretion of prodefensin, and interfered with defensin accumulation in cytoplasmic granules. These effects were reproduced by the smaller deletion Δ_{40-51}, which contains a highly conserved secondary structure. Therefore, propiece residues 40–51 appear to be essential for subcellular trafficking and sorting of human neutrophil α-defensins (Liu and Ganz 1995).

β-defensins in animal leukocytes

Instead of containing α-defensins like those found in human, rabbit, rat, hamster, and guinea pig neutrophils, bovine neutrophils contain an impressive array of β-defensin peptides (Selsted *et al.* 1993). Whereas myeloid α-defensins have free amino termini, about half of the β-defensins isolated from bovine neutrophils had a pyroglutamate residue at their amino terminus. This moiety characteristically results from enzymatic modification of an amino-terminal glutamine residue and could confer resistance to proteolytic cleavage by local proteases or alter other biologic properties. The β-defensin peptides from neutrophils manifest antibacterial activity against gram-positive and gram-negative bacteria, but because different *in vitro* assay conditions were used, direct comparison of the relative activity of bovine epithelial and neutrophil β-defensins is not yet possible.

β-defensins ("gallinacins") were also isolated from the leukocytes of chickens (Harwig *et al.* 1994) and turkeys (Evans *et al.* 1994). Two of the three chicken gallinacins differed at only three amino-acid residues and had comparable antibacterial and antifungal activities. The third gallinacin differed at over half of the amino acids and exerted antibacterial activity, but lacked the antifungal activity seen with the other two (Harwig *et al.* 1994). The β-defensins of turkeys showed good activity against *C. albicans*, *Salmonella enteriditis*, and *Campylobacter jejuni*, but were relatively ineffective against *Pasteurella multocida* at 16 μg/ml, the highest peptide concentration tested. They did not neutralize infectious bronchitis virus, an enveloped coronavirus (Evans *et al.* 1995). In normal chickens, the expression of gallinacin-3 was especially prominent in the tongue, bursa of Fabricius, and trachea. It also occurred in other organs, including skin, esophagus, air sacs, large intestine, and kidney. The tracheal expression of gallinacin-3 increased significantly in chickens experimentally infected with *Haemophilus paragallinarum* (Zhao *et al.* 2001).

α-DEFENSINS IN ENTERIC PANETH CELLS

Role of endogenous intestinal antibiotics

Despite continual entry of microorganisms from swallowed food and oral secretions, the normal small intestine contains only a sparse resident flora. Why? Although multiple factors contribute, the ability of intestinal enterocytes and Paneth cells to secrete antimicrobial molecules is likely to play a significant role. Through their differential antimicrobial activities, these molecules could contribute to selecting the normal intestinal flora, which may afford protection from pathogenic bacteria that enter the intestine. Since the intestinal epithelium stem cells reside in the intestinal crypts, secretion of Paneth cell defensins into the crypt lumen **(Figure 6.3)** could provide a protective barrier for this vital proliferative compartment.

Identification of enteric α-defensins in Paneth cells

Paneth cells, first described over 100 years ago, are located at the base of the crypts of Lieberkühn. They are more abundant in the region of the ileum and jejunum and less so in the duodenum. The high density of Paneth cells in the distal

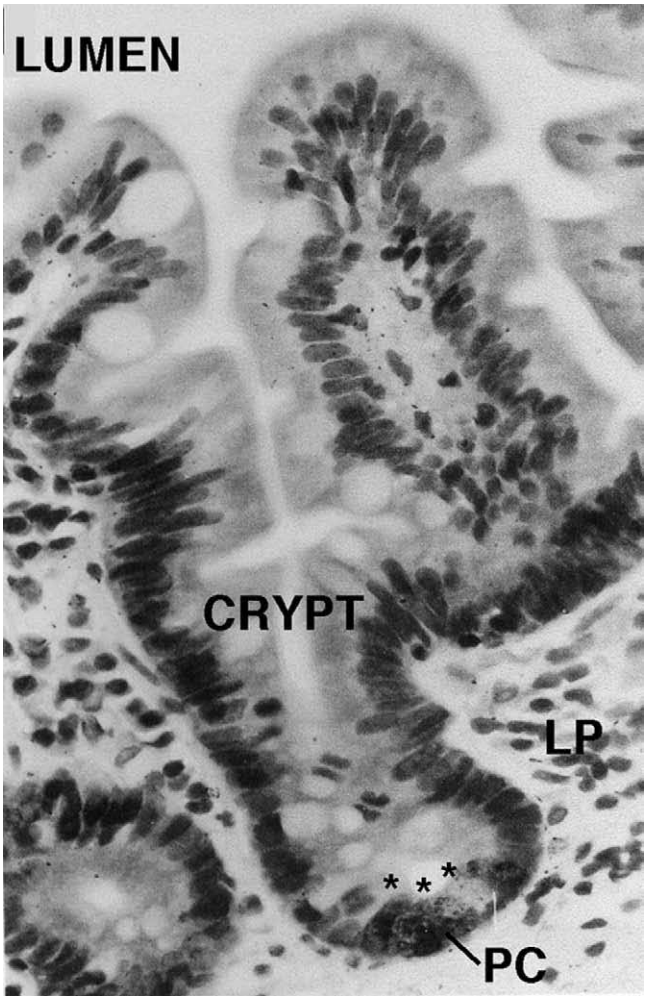

Fig. 6.3. Human small intestine, immunostained for HD-5. A longitudinally cut tubular crypt is prominent and contains several Paneth cells (PC) that contain numerous large, immunoreactive granules (asterisks). The intestinal lumen and the lamina propria (LP) have been labeled. Reproduced from Porter *et al.* (1997a) with permission.

small intestine might signify a role in preventing adverse consequences from reflux of colonic flora into the ileum. Paneth cells contain an extensive endoplasmic reticulum and Golgi network and are filled with numerous, apically located eosinophilic secretory granules whose secretion is stimulated by cholinergic agonists or the entry of bacteria or lipopolysaccharide into the intestinal lumen (Satoh 1988; Qu *et al.* 1996b).

Ouellette and colleagues (Ouellette *et al.* 1989) identified an α-defensin among several prominently expressed RNA messages in the postnatal mouse small intestine. Because this mRNA was expressed in Paneth cells at the base of small intestinal crypts, the corresponding peptides were named "cryptdins." Cryptdin mRNA levels were normal in germfree and nude mice, suggesting that expression was independent of T-cell signals or acquisition of the intestinal flora. Independently, two α-defensin genes (*HD-5* and *HD-6*) expressed in human Paneth cells were detected (Jones and Bevins 1992, 1993). In contrast to the six characterized murine enteric defensin genes, which had very high (85%) nucleotide similarity, *HD-5* and *HD-6* were not as closely related, presumably having duplicated and diverged before their murine counterparts (Bevins *et al.* 1996). The striking difference in murine and human enteric defensin gene numbers remains enigmatic. Paneth cells also secrete larger antimicrobial polypeptides, including lysozyme and secretory phospholipase A2 (see sections on "Identification of enteric α-defensins in Paneth cells" and "Developmental regulation of enteric α-defensin expression" in this chapter).

Antimicrobial activity of enteric α-defensins

Native mature defensins (cryptdins) have antibacterial and activity comparable with myeloid defensins (Selsted *et al.* 1992; Eisenhauer *et al.* 1992) and can kill the protozoan parasite *Giardia lamblia* (Aley *et al.* 1994). Recombinant human small intestinal defensin HD-5 was effective against *Listeria monocytogenes*, *S. typhimurium,* and *C. albicans* (Porter *et al.* 1997b; Ghosh *et al.* 2002). It showed minimal cytotoxic activity against human intestinal cell lines (Porter *et al.* 1997a).

Developmental regulation of enteric α-defensin expression

Morphologically identifiable Paneth cells appear in the mouse intestine immediately before birth. Thereafter, murine α-defensin mRNA levels and defensin immunoreactivity increase gradually and reach adult levels by the fourth postnatal week, paralleling expansion of the Paneth cell population. Mice reared in a germfree environment express comparable levels of enteric α-defensins (Ouellette *et al.* 1989; Putsep *et al.* 2000). In humans, a sensitive RT-PCR assay can detect mRNA encoding human enteric defensins HD-5 and HD-6 at 13.5 weeks' gestation, close to the time that morphologically distinguishable Paneth cells can be identified by electron microscopy. At approximately 24 weeks' gestation, HD-5 and HD-6 mRNA was detected in Paneth cells of the small intestinal crypt by *in situ* hybridization. By Northern blot analysis, the levels were approximately 100-fold lower than in adults. The ability to detect human enteric defensin mRNA prenatally indicates that its expression is at least partially constitutive and governed by a developmental program that operates without direct stimulation by microbes or their products.

Like their hematopoietic counterparts, intestinal α-defensins are initially synthesized as prepropeptides. However, unlike the myeloid defensins, Paneth cell defensins are stored as propeptides, exclusively so in humans (Porter *et al.* 1998; Ouellette *et al.* 2000; Ghosh *et al.* 2002). The post-translational processing of these epithelial α-defensins appears to be an important regulatory step in the generation of bioactive peptides in the small intestine. However, the pathways of processing appear to be quite different in mice and humans. In mice, the matrix metalloprotease matrilysin (matrix metalloproteinase 7) has been identified as

an essential enzyme in the processing of small intestinal α-defensins (Wilson *et al.* 1999). In humans, the orthologous small intestine α-defensins are processed to mature peptides during or after secretion into the small intestinal lumen by the serine protease trypsin (Ghosh *et al.* 2002). In both species, the proteases mediating this α-defensin processing are expressed by Paneth cells.

The role of Paneth cell defensins

The hypothesis that intestinal defensins contribute to the regulation of microbial flora in the small intestine received support from studies in matrilysin-deficient mice. Matrilysin (matrix metalloproteinase 7) is expressed specifically in Paneth cells. In mice with a targeted disruption of the intestinal prodefensin-processing protease, matrilysin, Paneth cell defensin precursors were not processed to active mature peptides. The mice had increased susceptibility to intestinal infections with gram-negative organisms (Wilson *et al.* 1999). Detailed analysis of secretions from the isolated crypts of normal and matrilysin-deficient mice indicated that the antimicrobial activity of crypt secretions was largely the result of defensins and that it was greatly impaired in matrilysin-deficient mice (Ayabe *et al.* 2000).

In another model, the transgenic expression of human defensin-5 in the Paneth cells of mice increased their resistance to orally administered *S. typhimurium* but, as might be expected, did not protect against infection by the peritoneal route (Saltzman *et al.,* 2003). In the aggregate, these models point to a central role for Paneth cell defensins as regulators of small intestinal flora and as enteroprotective molecules.

EPITHELIAL β-DEFENSINS

Bovine "tracheal antimicrobial peptide" (TAP) and other animal β-defensins

Bovine tracheal extracts contain an abundant peptide called "TAP," or tracheal antimicrobial peptide, that exhibited antimicrobial activity *in vitro* against *Klebsiella pneumoniae*, *S. aureus,* and *C. albicans* (Diamond *et al.* 1991). Sequence analysis revealed similarity of this molecule to defensins expressed in leukocytes and to its designation as the first epithelial β-defensin. TAP expression was found in the pseudostratified columnar epithelial cells of conducting airway and nasal mucosa (Diamond *et al.* 1993). A hallmark of TAP expression, like several more recently described epithelial β-defensins of humans and mice, is inducible expression in response to bacterial LPS and some inflammatory cytokines (Diamond *et al.* 1996; Russell *et al.* 1996; Diamond *et al.* 2000). A canonical NF-kB recognition site in the 5′-flanking region of the *TAP* gene was important in the transcriptional regulation of this gene by bacterial products (Diamond *et al.* 2000). *In vivo* inoculation of pathogenic bacteria into the bovine lung caused a rapid and dramatic increase in β-defensin expression in the conducting airway epithelium, which was not seen in the adjacent control lobe (Stolzenberg *et al.* 1997).

In bovine tracheal epithelial cells, β-defensin induction by LPS appears to be dependent on epithelium-derived CD14, a well-characterized LPS-binding protein (Diamond *et al.* 1996). This suggests that local expression of CD-14 might provide epithelial cells with the capacity to recognize bacterial products at mucosal surfaces and initiate local defense responses such as antimicrobial peptide production.

Several additional sites of β-defensin expression were identified in cattle, including squamous epithelial cells of the tongue (Schonwetter *et al.* 1995) and epithelial cells of the small intestine and colon (Tarver *et al.* 1998). mRNA encoding a bovine lingual β-defensin ("LAP") was markedly increased in epithelia surrounding naturally occurring tongue lesions, suggesting an integral role for antimicrobial peptides in local inflammatory responses. The antimicrobial activity of LAP and TAP was very similar, and LAP expression was enhanced in bovine trachea after LPS or TNF-α application (Russell *et al.* 1996). In mice, epithelial expression of β-defensins is also observed in the mucosal epithelium of the respiratory and digestive tracts (Morrison *et al.* 1999; Bals *et al.* 1999; Jia *et al.* 2000).

Similar to humans, mice express a noninducible β-defensin in kidney tubule cells and mucosal epithelia of several organ systems (Huttner *et al.* 1997). Recent studies of mice rendered deficient in this epithelial β-defensin through targeted homologous recombination have shown a minimal phenotype (Moser *et al.* 2002; Morrison *et al.* 2002). Further studies of these mice may be necessary to elucidate the functional role of this β-defensin.

Human epithelial β-defensins

A systematic characterization of the peptides present in human blood ultrafiltrates provided the first evidence for the existence of *HBD-1*, the first characterized human β-defensin (Bensch *et al.* 1995). *HBD-1* shared the nine invariantly conserved amino acids present in β-defensins from bovine and avian cells. Although free *HBD-1* was present in nanomolar concentrations in human plasma, an abundant *HBD-1* message occurred in the kidney and vagina (Bensch *et al.* 1995). Several *HBD-1* peptides, generated by differential posttranslational proteolysis at the N-terminus, were the predominant cationic peptides in urine. When tested, using urine as a growth medium, they displayed antibacterial activity against *E. coli. In situ* hybridization and immunostaining localized their site of production to the distal tubules of the kidney as well as the various epithelia of the female genital tract (Valore *et al.* 1998).

HBD-1 is also present in human conducting-airway epithelia (McCray and Bentley 1997) as well as in epithelia of many other tissues and in certain glands (Zhao *et al.* 1996). Expression of *HBD-1* by lung epithelia is developmentally regulated, in a manner reminiscent of the α-defensins found in rabbit lung macrophages (McCray and Bentley 1997). Circumstantial evidence implicates the impaired function of *HBD-1*, consequent to high bronchial fluid salinity (Smith *et al.* 1996), as a contributing factor in respiratory tract colonization by *P. aeruginosa* in cystic fibrosis patients (Goldman *et al.* 1997).

HBD-2 was first detected as an abundant cationic peptide and an antimicrobial component in the flaking epidermis of patients with psoriasis (Harder *et al.* 1997). It is produced by differentiated keratinocytes in the epidermis under the influence of inflammatory signals, principally IL-1 (Liu *et al.* 1998, 2002, 2003) and secreted in lamellar bodies into the space between keratinocytes, along with the lipids that make the epidermis impermeable to water (Oren *et al.*, 2003). There, *HBD-2* reaches concentrations sufficient for antimicrobial activity (Liu *et al.* 2002). *HBD-2* is active against a broad range of microbes, with the significant exception of the skin pathogen *S. aureus* (Harder *et al.* 1997; Liu *et al.* 2002).

HBD-3 was detected more or less simultaneously by genomic methodologies (Jia *et al.* 2001; Garcia *et al.* 2001a) and by direct extraction of the peptide from psoriatic skin (Harder *et al.* 2001). *HBD-3* is one of the most cationic defensins known, with a charge of +11 at neutral pH. Unlike *HBD-2*, *HBD-3* is active against *S. aureus* and even *B. cepacia*, at least under low ionic strength conditions. The skin and the tonsils appear to have the highest levels of expression, but *HBD-3* is detectable in other epithelial and nonepithelial tissues as well. The mechanisms of its regulation have not yet been reported.

Genomic analysis indicates that many additional human β-defensin genes and related genes exist, but they remain to be characterized at the peptide level (Schutte *et al.* 2002).

EVOLUTIONARY CONSIDERATIONS

The α-defensin and β-defensin families almost certainly arose by divergence from an ancestral premammalian defensin and not by convergent evolution. This conclusion is supported by the proximity of human α-defensin and β-defensin genes within 150 kb on chromosome 8p23 (Liu *et al.* 1997). Defensinlike molecules not assignable to either defensin family have been identified in invertebrates. Hemocytes of the horseshoe crab, *Tachypleus tridentatus,* contain an antibacterial peptide ("big defensin") whose C-terminal 37 residues have a cysteine structure resembling that of mammalian β-defensins and a primary sequence similar to rat myeloid α-defensins (Saito *et al.* 1995). When the unusually long, 35-residue amino-terminal extension of big-defensin was cleaved by tryptic digestion, both the defensinlike domain and the amino-terminal extension had antimicrobial properties. An extremely defensinlike antiviral peptide was also identified in the sea anemone, *Anemonia sulcata* (Driscoll *et al.* 1989).

The primary structural resemblance between avian β-defensins (gallinacins) and certain cytostatic (Marquardt *et al.* 1988) and myotoxic peptides in snake venoms **(Fig. 6.4)** also suggests that the progenitors of β-defensins had potential to become weapons of offense, as well as of host defense. The same can be said about the antimicrobial insect defensins, which show striking structural similarity to certain toxic peptides found in scorpion venom (Cociancich *et al.* 1993).

Given such findings, it is reasonable to postulate the antiquity of defensins as host-defense molecules. An evolutionary relationship between vertebrate defensins and the structurally more distant defensins of insects and plants is possible, but lacks convincing supporting data.

OTHER MUCOSAL DEFENSE MOLECULES

Calprotectin

Calprotectin, a member of the widespread calcium-binding S-100 protein family, is present in remarkably high concentration in the cytoplasm of human neutrophils (Brandtzaeg *et al.* 1995). The calprotectin molecule is composed of light (MRP8) and heavy (MRP14) subunits. Although not secreted from intact neutrophils, calprotectin release from dead and dying neutrophils creates high concentrations of the protein in inflammatory or abscess fluids and in the intestinal tract lumen of patients with inflammatory bowel disease. Calprotectin is also found in reactive tissue macrophages, in nonkeratinizing squamous epithelia, and in reactive epidermal cells. The C-terminal portion of calprotectin's heavy chain, MRP14, is identical to a peptide called neutrophil immobilizing factor, "NIF." Calprotectin concentrations of 50–250 μg/ml inhibit growth by *S. aureus*, S. *epidermidis,* and *E. coli.* Even lower concentrations (4–32 μg/ml) inhibit growth by *C. albicans*, possibly by depriving the *Candida* cells of zinc. Calprotectin purified from rat inflammatory peritoneal cells was markedly cytotoxic for mitogen-stimulated lymphocytes and for various tumor cell lines, in

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Myotoxin A          YKQCHKKGGHC---ICIPPSSDLGKMDCRWKWKCCKKGSG
                      ||    || |    +|     ||+||+ |    |||||+   |
SVGAP                SQCEQEGGFCRFLLCPSRTSDIGKLGCEPLWKCCKRWGG
                     | |     ||| || ||| |    ||   |      ||||
Gallinacin-1     GRKSDCFRKSGFCAFLKCPSLTLISGK--CSRFYLCCKR
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Fig. 6.4. From serpent's tooth to chicken soup. Myotoxin A is a myotoxic peptide from the venom of *Crotalus viridis viridis,* the prairie rattlesnake (Fox *et al.* 1979). SVGAP stands for snake venom growth arresting peptide (Marquardt 1988), and is described in U.S. patent 4774318. Gal-1 is the chicken β-defensin gallinacin-1, originally isolated from chicken heterophils (Harwig *et al.* 1994) and later found in chicken soup (Lehrer unpublished). The disulfide pairing patterns (1:5, 2:4, 3:6) of myotoxin A (Fox *et al.* 1979) and avian β-defensins (Harwig, unpublished) are identical.

which it induced apoptosis. Epithelial cell calprotectin might protect host cells from microbes that invade the cytoplasm directly or by lysing phagosomal membranes.

The murine MRP8 protein (also called CP10 or S100A8) is chemotactic for myeloid cells but this property is not shared by human calprotectin. Homozygous disruption of *MRP8* (*S100A8*) gene in mice causes embryonic death by day 9.5 but *MRP14* (*S100A9*)-deficient mice are viable and healthy (Manitz *et al.* 2003) even though their neutrophils lack both *MRP8*/*14* partners. Initial studies of these mice detected only very mild defects in neutrophil cytoskeletal organization and migration, but the activity of these neutrophils in microbicidal assays has not been reported.

Lysozyme (muramidase)

Lysozyme was discovered over 75 years ago by Fleming, who called it a "remarkable bacteriolytic ferment" and did not patent it—or penicillin, which he discovered a few years later. This very cationic 14.3-kDa protein enzymatically attacks the β1-4 glycosidic bonds between N-acetylglucosamine and N-acetylmuramic acid, which stabilize bacterial peptidoglycans. Gram-positive bacteria susceptible to the lytic action of lysozyme include *Bacillus subtilis*, *Bacillus megaterium,* and *Micrococcus luteus* (*nee lysodeicticus*). Lysozyme also exerts bactericidal activity by nonenzymatic, nonbacteriolytic mechanisms. It is a remarkably abundant component of phagocytic leukocytes, and also of tears, saliva, and many other secretions. The detection of abundant lysozyme in Paneth cell granules yielded the first clue that these intestinal epithelial cells were important in host defense (Peeters and Vantrappen 1975). Most gram-negative bacteria are resistant to lysozyme, except under very low ionic strength conditions. This is caused largely by the protective effects of their outer membrane, which covers and masks their peptidoglycan layer. Lysozyme may function most effectively in conjunction with other antimicrobial factors, especially those capable of permeabilizing the outer membrane.

Recent experiments in mice deficient in lysozyme-M (Ganz *et al.,* 2003) showed delayed killing of the lysozyme-sensitive *M. luteus*, but the major abnormality in these mice was the highly exaggerated inflammatory response to these bacteria and to peptidoglycan. Peptidoglycan is a potent inflammatory stimulus, in part through its binding to TLR-2 and by peptidoglycan recognition proteins. Whatever lysozyme may contribute to antibacterial activity, it appears to be critically important for eliminating bacterial peptidoglycan, thus extinguishing the strong signal to innate immunity and inflammation generated by this characteristic bacterial macromolecule.

Lactoferrin

Lactoferrin is a 78-kDa single-chain glycoprotein that can bind one or two molecules of ferric iron. It is found in various secretions, including tears, milk, and semen, and also in the secretory granules of neutrophils. Intestinal cells and mononuclear phagocytes have surface receptors for lactoferrin, but do not synthesize it. Certain bacteria (e.g., *N. gonorrhoeae, Neisseria meningitidis,* and *Moraxella catarrhalis*) also possess lactoferrin receptors and use them to acquire iron. Lactoferrin or lactoferricin-treated gram-negative bacteria release LPS, develop electron-dense "membrane blisters," and become sensitized to lysozyme—all consistent with outer membrane damage. Lactoferrin binds bacterial lipopolysaccharide with high affinity and also binds various other proteins. Exposure of lactoferrin to pepsin (its normal fate when ingested in milk) releases an antimicrobial polypeptide, "lactoferricin" comprising the amino-terminal lactoferrin residues (Tomita *et al.* 1994). Lactoferricin kills many bacteria and *C. albicans*, but is ineffective against *Proteus* and *Serratia* species and *P. cepacia*. Lactoferrin also inhibits microbial adherence to and invasion of epithelial cells.

Lactoferrin has a higher affinity for iron than its plasma homolog transferrin, and its iron binding is less affected by acid pH. These characteristics may allow it to function as an iron-sequestering substance in mucosal secretions and inflammatory fluids. Because iron is an essential metal for all living organisms, sequestration of iron is an effective means of inhibiting microbial growth. Iron starvation also interferes with bacterial biofilm formation. Even under conditions that otherwise promote biofilm formation, lactoferrin-exposed bacteria remain in the planktonic form, presumably allowing the more mobile bacterial population to disperse and reach iron sources (Singh *et al.* 2002).

Secretory phospholipase A_2 (PLA_2)

Phospholipase A_2 enzymes remove fatty acids from the middle (*sn*-2) carbon atom of phosphoglycerides. Several phospholipase A_2 enzymes occur in mammalian cells, but only the 14-kDa secretory PLA_2 ($sPLA_2$) has potent microbicidal properties, principally against gram-positive bacteria (Harwig *et al.* 1995; Weinrauch *et al.* 1996). $sPLA_2$ differs from pancreatic PLA_2 structurally and in preferring phospholipids prominent in bacterial membranes [e.g., phosphatidylglycerol (PG) and phosphatidylethanolamine (PE)] over phosphatidylcholine as substrates. PLA_2 is present in granules of human neutrophils and small intestinal Paneth cells. $sPLA_2$ is released from neutrophils exposed to secretagogues, such as phorbol esters or calcium ionophores, and by Paneth cells exposed to cholinergic agonists, bacteria, or LPS. Serum $sPLA_2$ levels are greatly elevated in patients with sepsis and rise sharply within 3 hours after the experimental administration of lipopolysaccharide to uninfected patients. High concentrations of sPLA are also present in human tears. The antimicrobial specialization of this phospholipase is probably a result of its unusually high net positive charge and a cluster of positively charged residues near its N-terminus that facilitate its interaction with bacteria and access to its phospholipid target (Weiss *et al.* 1991).

Proteinase inhibitors

SLPI is a 107-aa (12.7-kDa) nonglycosylated peptide with a bipartite structure. It is very abundant in many human secretions, including seminal plasma, cervical mucus, nasal

secretions, and tears. SLPI has received many alternative names, including human seminal plasma inhibitor-I (HUSI-I), cervix uteri secretion inhibitor (CUSI), bronchial secretory inhibitor (BSI), and bronchial mucus inhibitor (MPI). Mice deficient in SLPI show impaired wound healing (Ashcroft *et al.* 2000; Zhu *et al.* 2002), but their response to infection has not been reported yet. *In vitro,* SLPI shows low-level activity against bacteria and fungi (Hiemstra 2002) and may also have anti-HIV activity. If the high concentrations of SLPI in many epithelial secretions compensate for its low intrinsic potency, its antimicrobial activity could be biologically important.

SLPI is synthesized as a 132-aa prepropeptide with a 25-aa signal peptide. Each of its two structurally similar domains contains four intradomain disulfide bonds in the "four-disulfide-core" pattern also present in wheat germ agglutinin and neurophysin. The C-terminal domain of SLPI (residues 55–107) shows homology to the second domain of chelonianin, a basic protease inhibitor from Red Sea turtle, and is responsible for the molecule's prominent antiprotease activity (Masuda *et al.* 1995). SLPI holoprotein and its N-terminal domain (residues 1–54) have microbicidal properties (Hiemstra *et al.* 1996). Other protease inhibitors with antimicrobial properties include aprotinin, a 58-aa protease inhibitor in bovine mast cells (Pellegrini *et al.* 1996), and ENAP-2, a four-disulfide core protein found in equine granulocytes (Couto *et al.* 1992). Multifunctionality may be a common attribute of other proteins involved in host defense.

Antimicrobial ribonucleases

Eosinophils, as well as other myeloid and epithelial cells, contain cationic antimicrobial proteins belonging to the ribonuclease family (Rosenberg and Domachowske 2001). In humans, eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) are among the principal components of eosinophil granules. ECP is broadly antimicrobial (Lehrer, 1989b) and toxic to helminths (Hamann *et al.* 1987, 1990), but EDN is nearly inactive in these assays. Both ECP and EDN show antiviral activity (Rosenberg and Domachowske 2001) against respiratory RNA viruses: respiratory syncytial virus (RSV) and a related murine virus. New members of the antimicrobial ribonuclease family likely to be important in defending body surfaces include RNase 7, an abundant component of human epidermis (Harder and Schroder 2002) and angiogenin 4, an antimicrobial protein of murine Paneth cell granules (Hooper *et al.* 2003). The specific role of these proteins in host defense remains to be defined.

BPI

Bactericidal permeability-increasing protein (BPI) (Elsbach and Weiss 1998) is found in the azurophil (primary) granules of human and rabbit neutrophils, but like defensins, may be absent from mouse neutrophils. It is a 55-kDa cationic protein and a member of a family of lipid-binding proteins, two of which, BPI and lipopolysaccharide-binding protein (LBP) avidly bind to bacterial lipopolysaccharide. *In vitro,* BPI is specifically active against selected gram-negative bacteria at concentrations as low as nanomolar, and this activity is wholly contained in a 25-kDa amino terminal fragment. The mechanism of activity of BPI against bacteria depends on the initial high-affinity interaction with LPS in the outer membrane. The resulting rapid permeabilization of the outer membrane is followed by a slower process that culminates in the disruption of the inner membrane of gram-negative bacteria with attendant loss of viability (Wiese *et al.* 1997). More recently, BPI was also identified as a component of human eosinophil granules and as a lipoxin-inducible protein in mucosal epithelia (Canny *et al.* 2002). *In vitro* studies suggest that BPI contributes to the killing of gram-negative bacteria in isolated epithelia and neutrophils.

Cathelin-associated peptides ("cathelicidins")

The neutrophils of humans, pigs, cattle, sheep, mice, and other mammals contain a variety of structurally diverse antimicrobial peptides collectively called "cathelicidins." These peptides were grouped together because they are synthesized at the carboxy-terminal portion of a precursor containing a highly conserved domain, approximately 100 amino acids long, called "cathelin" (Zanetti *et al.* 1995; Ganz and Weiss 1997). Some of these peptides have amphipathic α-helical structures, others form compact, defensin-like β-sheets stabilized by intramolecular cystine disulfide bonds, and still others are remarkably rich in proline, arginine, or tryptophan residues. The genes for cathelicidins contain four exons, the first three of which encode most of the conserved cathelin domain. Exon 4 specifies the last few cathelin residues, including the cleavage site and the mature peptide. The 5′ flanking sequences of this gene family contain motifs for binding NF-κB, IL-6, GM-CSF, and NF-1, suggesting that synthesis responds to mediators generated early during infection.

The sole known human cathelicidin is named hCAP18 or FALL39/LL37 (Agerberth *et al.* 1995; Cowland *et al.* 1995; Larrick *et al.* 1995). Unlike defensins, which are fully processed before they are stored in the azurophil granules of the neutrophil, the human cathelicidin peptide is stored in the specific (secretory) granules as hCAP18, a cathelin-containing, 140-residue, 17-kD propeptide. During or after secretion, hCAP18 undergoes proteolytic processing by proteinase 3 (Sorensen *et al.* 2001) to liberate LL-37, the 37-residue, α-helical antimicrobial peptide at its C-terminus (Gudmundsson *et al.* 1996). The analogous proteolytic processing of bovine cathelicidins (proBac5 and proBac7) and porcine cathelicidins (proprotegrins 1–3) is mediated by trace amounts of neutrophil elastase (Panyutich *et al.* 1997; Scocchi *et al.* 1992).

mRNA for hCAP18 is found in testis (Malm *et al.* 2000), inflamed keratinocytes (Frohm *et al.* 1997; Agerberth *et al.* 1995), and airway epithelia (Bals *et al.* 1998). *In vitro,* LL-37 displayed both LPS binding (Hirata *et al.* 1995) and microbicidal activities (Agerberth *et al.* 1995) against *E. coli*. The abundance of LL-37 in neutrophil specific granules is about a third that of lactoferrin or lysozyme, the principal proteins of specific granules (Borregaard *et al.* 1995).

In addition to microbicidal activity, certain cathelicidins also have chemoattractant activity. For example, the human

cathelicidin LL-37 is chemotactic for neutrophils, monocytes, and T cells, but not for dendritic cells. This activity is likely mediated through binding to the formyl-peptide receptorlike 1 receptor (De *et al.* 2000). The proline-rich porcine cathelicidin PR-39 can induce upregulation of syndecans, heparan sulfate proteoglycans involved in the repair process at the site of skin wounds (Gallo *et al.* 1994).

Although the pig has a large number of cathelicidin genes, the most active antimicrobial peptides in porcine skin wounds are protegrins, secreted from neutrophils as proprotegrins and activated by proteolytic cleavage by neutrophil elastase (Panyutich *et al.* 1997). *In vitro*, inhibition of neutrophil elastase by specific inhibitors blocked the conversion of proprotegrins to protegrins and largely ablated the stable antimicrobial activity secreted by porcine neutrophils (Shi and Ganz 1998). The application of neutrophil elastase inhibitor to porcine wounds decreased the concentration of mature protegrin in wound fluid and impaired the clearance of bacteria from wounds. The deficit could be restored by supplementing the wound fluid with synthetic protegrin *in vitro* or *in vivo* (Cole *et al.* 2001). Thus protegrins act as natural antibiotics that contribute to the clearance of microbes from wounds.

Strong evidence for the antibacterial function of cathelicidins in the skin comes from studies of dermal infections in mice with an ablated gene for CRAMP (cathelin-related antimicrobial peptide). CRAMP, normally the main murine cathelicidin (Nizet *et al.* 2001), is the murine homolog of human hCAP18. In CRAMP-knockout mice, the skin lesions caused by experimental Group A *Streptococcus* inoculation were larger lesions and had higher numbers of viable bacteria than those seen in control mice. As in pigs, infiltrating neutrophils appeared to be the chief source of cathelicidins in inflamed skin. Epithelial cells also produced cathelicidins, but it is not clear if their production was quantitatively significant. How or if epithelial cathelicidins undergo proteolytic processing remains to be defined.

ACKNOWLEDGMENTS

Many of our previous and ongoing studies have been supported by grants from the National Institutes of Health, including: AI 32234, 32738, and 50843 to C. B.; AI 37945 and 22837 to R. I. L.; and HL46809, AI46514, and AI48167 to T. G. The continuing support of the Will Rogers Institute is gratefully acknowledged. We thank Ken Miyasaki for his artistic rendering of the human defensin dimer, all our past and present collaborators for putting up with us, and antimicrobial peptides for being there.

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