Old Herborn University Seminar Monograph 15:
Probiotics: bacteria and bacterial fragments as immunomodulatory agents.
Editors: Peter J. Heidt, Tore Midtvedt, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 5-13 (2002).

DEFENSINS AND INNATE IMMUNITY OF THE MAMMALIAN SMALL INTESTINE

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SUMMARY

The release of gene-encoded antimicrobial peptides by epithelial cells is thought to contribute to innate mucosal immunity. Defensins are a predominant class of such antimicrobial peptides in mammals. These cysteine-rich cationic peptides have antibiotic activity against a wide range of bacteria and other microbes. In the mammalian small intestine, Paneth cells at the base of the crypts of Lieberkühn secrete defensins and other antibiotic proteins. These Paneth cell antimicrobials are proposed to have several overlapping functions. First, they likely help to protect the epithelial stem cells from noxious microbes. The stem cells, which reside at the neck of the crypt, are responsible for continual renewal of cells necessary for maintained integrity of the surface epithelium lining the villi and crypts. Second, defensins and other Paneth cell products likely interact with bacteria that exist in the intestinal lumen. Based on relative sensitivity to these antimicrobials, the composition of the enteric microbial flora might be influenced. Third, enteric defensins may regulate the numbers of colonising microbes in the small intestine. Forth, enteric defensins may contribute to defence from food and water borne pathogens in the intestinal lumen. Further defining the contributions of Paneth cell defensins to innate defence should improve our understanding of normal small bowel function. Given that microbial products stimulate Paneth cell secretion, it is possible that the mechanism of action of probiotic agents may, in part, involve modulating Paneth cell secretion.

INNATE HOST DEFENCE OF THE SMALL INTESTINE

The mucosal surface of the mammalian small intestine is remarkable from the perspective of host defence. Its life-sustaining physiological function requires that these sites maintain direct contact with the external world via the lumen. The surface area of this mucosa is amplified anatomically by its many folds, villi and microvilli that increase the luminal absorptive area (Figure 1). The structural adaptations that have evolved to satisfy requirements for adequate nutrient absorption also predict a host vulnerability, because they increase the opportunity for microbes to establish invasive infections. In addition, the requirements of efficient nutrient absorption also place limits on the barrier components of host defence. Unlike many other surface epithelia, the intesti-

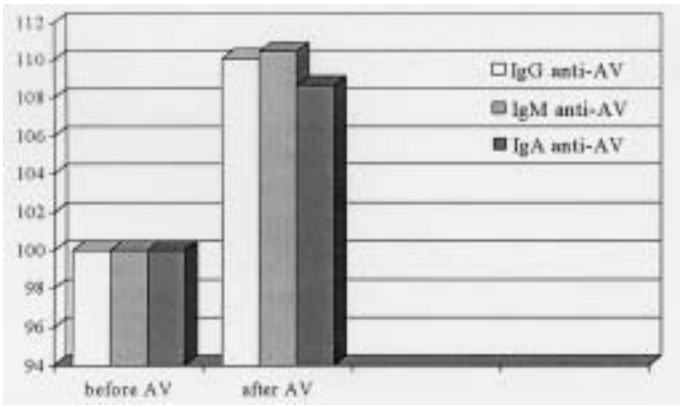

Figure 1: Microbial challenges in the alimentary tract - a schematic diagram.
The alimentary tract of humans and other non-ruminant mammals are confronted with microbial challenge (designated as dots) from abundant quantities of microbes in a heavily colonised colon and from variable quantities of microbes in food and water sources. In contrast, the small intestine has relatively low level of colonisation. The surface area of the small intestine is large, amplified by mucosal folds, villus projections and microvilli. Paneth cells are found along the length of the small intestine. These secretory epithelial cells, armed with defensins and other antibiotic peptides, reside in the crypts of Lieberkühn.
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nal epithelia are comprised of only a single layer of cells. While the complications that often follow antibiotic therapies offer support for the notion that colonising symbiotic microbes contribute to host defence against pathogens, in the absence of effective defence mechanisms even these microbes could rapidly multiply and overwhelm the mammalian host. Thus, the epithelium of the small bowel must execute its digestive and absorptive roles while inhibiting most microbes from establishing themselves as significant resident populations.
The architecture of the small intestinal epithelium is characterised by villi and crypts (Figure 2). This epithelium is replaced continually by a process involving stem cell mitosis, cellular differentiation and migration, apoptosis and ultimately exfoliation. Stem cells, which reside in the neck of the intestinal crypts, replicate to maintaining a continuous supply of new epithelial cells required to repopulate the villi and crypts (*Potten*, 1998; *Booth* and *Potten*, 2000). An interruption of this stem cell replication would have important consequences for the maintenance of the normal digestive epithelium and could generate portals of entry for luminal bacteria. Therefore, mechanisms that protect crypts against bacterial overgrowth and infection are also key elements of small intestinal defence.

Innate immunity (also termed natural immunity) encompasses a complex array of defence mechanisms (*Janeway* and *Travers*, 1997). At mucosal surfaces the innate defence system generally employs two broad and overlapping strategies that are central to effective defence: minimise microbial adherence and create a hostile environment for potential pathogens. In the small bowel, examples of innate defences include:

i) *physical processes*, such as peristalsis and the shedding of epithelial cells,

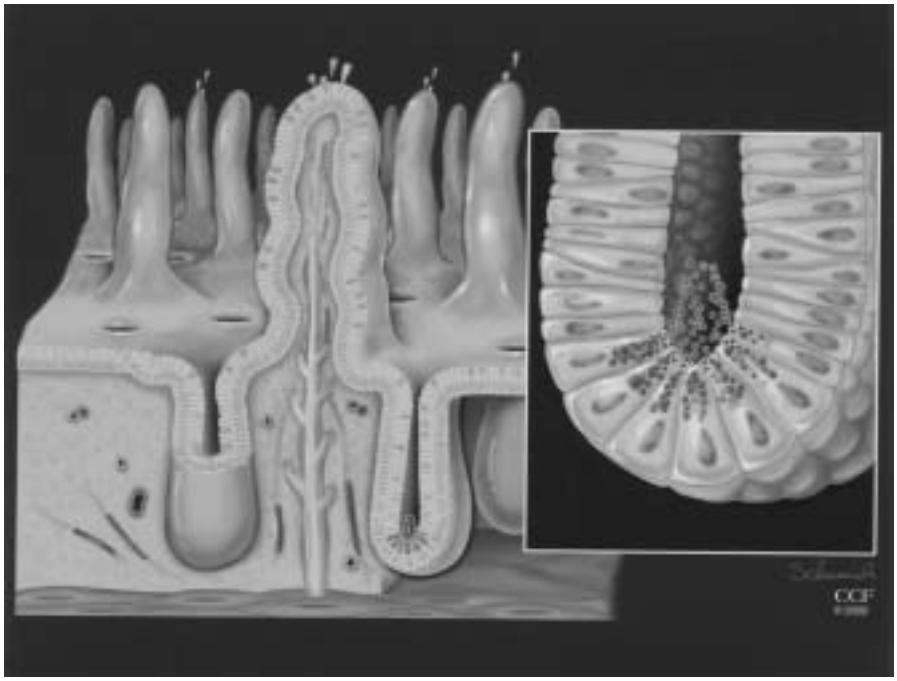

Figure 2: Small intestinal crypt architecture.
An artist's view of the small intestinal villi and crypt relationship (*Ganz*, 2000). Stem cells reside at the neck of the small intestinal crypt and divide. Some of their progeny cells migrate upward toward the villi or while others migrate deeper towards the base of the crypt. Those cells migrating towards the villus tips undergo cellular differentiation into absorptive enterocytes, goblet cells or entero-endocrine cells. The life span of these villus cells from their origin in the crypt, through migration and differentiation, until apoptotic death and exfoliation into the lumen is approximately 2-5 days. The other cells, which descend towards the crypt base, differentiate into Paneth cells. Paneth cells have life span of several weeks. Inset. Paneth cells release secretory vesicles into the narrow lumen of the crypt (*Ganz*, 2000). These secretions contain α-defensins, lysozyme and secretory phospholipase A_2. This potent antimicrobial cocktail is proposed to have several host defence functions as discussed in the text.
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ii) *chemical barriers*, such as mucus and various peptides and proteins, and
iii) *cellular processes*, such as phagocytosis.

In the small intestine, activities associated with the process of digestion may also contribute to innate mucosal defences. For example, gastric acidity, digestive enzymes, and bile salts may contribute effectively to the inhibition of microbial growth. Innate host defence mechanisms interface with the acquired (also termed clonal or adaptive) immune responses mediated by lymphocytes (*Fearon*, 1997; *Medzhitov* and *Janeway*, 1997). Clearly, mammals commit extensive resources to lymphocyte mediated adaptive immune functions in the intestinal compartment. Prominent examples include humoral immunity via B cell-mediated release of secretory IgA which traverses the epithelium to the gut lumen, and cell-mediated immunity via intraepithelial T-cells of villi (*Neutra* et al., 1996). However, in contrast to the lymphocyte-mediated immune system,

where an effective response involves both gene rearrangements and clonal selection developed over a period of days, the innate system remains ever-ready or immediately inducible. Therefore, innate host defence mechanisms, including both physical and chemical factors, are thought to provide immediate protection against the threat of colonisation and infection by deleterious microorganisms. This essay will focus on one component of innate immunity, defensins, a group of antimicrobial peptides that contribute to defence in the small intestine and other body surfaces. For more in-depth discussion of defensins the interested reader is directed to several reviews (*Martin* et al., 1995; *Lehrer* et al., 1998; *Ganz*, 1999).

ANTIMICROBIAL PEPTIDES IN INNATE HOST DEFENCE

Antimicrobial peptides contribute to host defence in a wide variety of settings, including plant seeds, arthropod haemolymph, and mammalian phagocytes and epithelia (*Zasloff*, 1992; *Boman*, 1995; *Hancock* et al., 1995; *Lehrer* et al., 1998; *Huttner* and *Bevins*, 1999). Unlike many other antibiotics in nature, which are secondary metabolites, antimicrobial peptides are encoded by conventional genes. There is considerable structural diversity in the many dozens of antimicrobial peptides, ranging from simple, alpha-helical linear molecules to molecules with beta-sheet conformation and multiple disulphide linkages. However, most of these peptides are cationic and amphipathic and they are generally active at micromolar concentrations against a broad range of microbes. Disruption of the target microbial membrane function is a typical feature of the mechanism action of most peptides investigated to date, and in some cases pore formation, membrane depolarisation, disruption of bacterial energy metabolism and interference with biosynthetic pathways have been observed.

Experimental evidence for the specific roles of antimicrobial peptides in host defence has been provided by experiments that assess the impact of ablation or augmentation of antimicrobial peptide production. Augmentation of antimicrobial peptide production in plants has been shown to increase their resistance to plant pathogens (*Epple* et al., 1997; *Fritig* et al., 1998). Experiments in Drosophila have demonstrated that ablation of pathways that induce the production of antifungal peptide drosomycin dramatically reduce survival after fungal infections (*Lemaitre* et al., 1996). Similar experiments in transgenic mice are a subject of intense effort in several laboratories. The multiplicity of antimicrobial peptides and redundancies in the innate and adaptive immune systems in mammals has presented challenges to the design of these experiments.

DEFENSINS

In mammals, defensins are one of the major families of antimicrobial peptides (*Lehrer* et al., 1998; *Ganz*, 1999). Characteristically, these peptides are 18 to 42 amino acids in length, have predominance of β-sheet conformation, are cationic in net charge and contain six cysteines that participate in three intramolecular disulphide bonds. Based on the spatial distribution of the cysteine residues, defensins are classified into three major groups termed α-, ß-, and

θ-defensins. The α-defensins were first identified in phagocytic leukocytes (*Ganz* et al., 1985) and later identified in mouse and human Paneth cells (*Jones* and *Bevins*, 1992; *Selsted* et al., 1992). In humans, two such Paneth cell α-defensins have been identified, HD-5 and -6 (*Jones* and *Bevins*, 1992; *Jones* and *Bevins*, 1993; *Mallow* et al., 1996; *Porter* et al., 1997; *Porter* et al., 1998). Analysis of α-defensin gene structure in several species reveals that all haematopoietic α-defensin genes have three exons, but the epithelial alpha-defensin genes have two. The ß-defensins were first isolated from trachea and neutrophils of cattle (*Diamond* et al., 1991; *Selsted* et al., 1993) and later in the mucosa of the airway, tongue, colon, kidney, skin, and gingiva in humans and in other species (*Diamond* and *Bevins*, 1998). In the digestive tract, ß-defensins have been found in the gingival epithelia, the tongue and the colon (*Schonwetter* et al., 1995; *Krisanaprakornkit* et al., 1998; *Tarver* et al., 1998; *Shi* et al., 1999). The single known θ-defensin, RTD-1, is a 2 KDa macrocyclic peptide that is found in phagocytic leukocytes of the Rhesus macaque (*Tang* et al., 1999). RTD-1 consists of an 18 amino acid, covalently closed circular polypeptide chain that is stabilised by three disulphide bonds. In humans, the genes that encode defensins are clustered in a few hundred kilobase segment of the short arm of chromosome 8 (8p23) (*Sparkes* et al., 1989; *Harder* et al., 1997; *Liu* et al., 1997), and the corresponding homologous genes are similarly clustered in the rodent genomes (*Ouellette* et al., 1989; *Huttner* et al., 1997; *Bals* et al., 1998; *Morrison* et al., 1998; *Jia* et al., 2000).

ANTIMICROBIAL PEPTIDES OF PANETH CELLS

In most mammals, including humans and rodents, Paneth cells occupy a position at the base of the crypts of Lieberkühn (*Trier* et al., 1967). Paneth cells have distinct morphology. They are intensely eosinophilic, with ultrastructural hallmarks of secretory cells, including an extensive endoplasmic reticulum and Golgi network (*Trier*, 1963). They secrete large secretory granules apically into the crypt lumen. Paneth cells are found from the duodenum to the ileum. Unlike other epithelial cells of the small intestine, which are short lived, Paneth cells have an average life span of approximately three weeks. Since Paneth cells develop prenatally during normal human ontogeny (*Mallow* et al., 1996) and are present in mice reared under germ-free conditions (*Ouellette* et al., 1989), their ontogeny does not depend on luminal bacteria or dietary constituents.

Identification of Paneth cell proteins has provided key insights into the biological role of these cells. Paneth cells of rodents and humans produce lysozyme (*Erlandsen* et al., 1974; *Peeters* and *Vantrappen*, 1975), secretory phospholipase A_2 ($sPLA_2$) (*Senegas-Balas* et al., 1984; *Harwig* et al., 1995), and α-defensins (*Jones* and *Bevins*, 1992; *Selsted* et al., 1992; *Porter* et al., 1997, 1998; *Cunliffe* et al., 2001), well-established antimicrobial proteins and peptides.

ANTIMICROBIAL ACTIVITIES OF PANETH CELL DEFENSINS

Mouse and human Paneth cell α-defensins are potent antimicrobial agents with selective activities against several varied microbial cell targets. For exam-

ple, HD-5 is active against a variety of bacterial species, including *L. monocytogenes*, *E. coli*, *S. typhimurium*, as well as the yeast-like fungus *C. albicans* (*Porter* et al., 1997). *In vitro* assays of cryptdins show that they are similarly microbicidal against *E. coli* ML35, *Staph. aureus*, and *S. typhimurium* (*Selsted* et al., 1992). In a structure-function analysis of mouse Paneth cell α-defensins, trophozoites of *Giardia lamblia* are were highly sensitive to two defensins, but far less sensitive to two others. Peptide amino acid residue position 15 was implicated in this activity, because the active defensins contain Arg at position 15 whereas the inactive defensins contain a Gly at this position (*Aley* et al., 1994).

PROPOSED FUNCTION(S) OF PANETH CELL DEFENSINS

The proposed physiological roles of small intestinal defensins may be grouped into 4 overlapping processes (Figure 3).

I) Enteric defensins may provide protection of the crypt and its stem cells from microbial invasion and parasitisation. Estimates from studies of isolated mouse crypts indicate that the concentration of defensins in the crypt at mM levels. In view of the well-documented antimicrobial activity of many defensins at concentrations in the μM range, it is reasonable to propose that an important function of Paneth cell defensins is crypt protection.

II) Enteric defensins may contribute to the factors that are selective for commensal bacteria. Because of selective sensitivity to their antimicrobial activity, the microbiological makeup of the intestinal flora may, in part, be governed by enteric defensins.

III) Enteric defensins may regulate the numbers of colonising microbes in the small intestine. Secretions from Paneth cells, which are present in highest numbers in the distal small intestine, contribute to an antimicrobial milieu that might prevent the abundance of colonic microbes from colonising the distal the small intestine in similar numbers. This function might help explain in part the perplexing observation that the bacterial load in the small intestine is estimated at 10^4 to 10^6 folds fewer microbes per gram of tissue than in the adjacent colon.

IV) Enteric defensins may contribute to defence from food and water borne pathogens in the intestinal lumen. Newly colonising microbes, including pathogens, will be confronted enteric defensins, and other Paneth cell antimicrobials. Given that parasympathetic (cholinergic) neural activity regulates numerous digestive functions throughout the alimentary tract, and that cholinergic stimulation also elicits Paneth cell secretion, we speculate that neurally mediated cholinergic stimulation that accompanies oral ingestion might induce Paneth cell secretion. This could equip the intestinal lumen with anticipatory effector molecules, including defensins, that could counter noxious microbes ingested my mouth.

These 4 possibilities are suggested to be overlapping and not mutually exclusive.

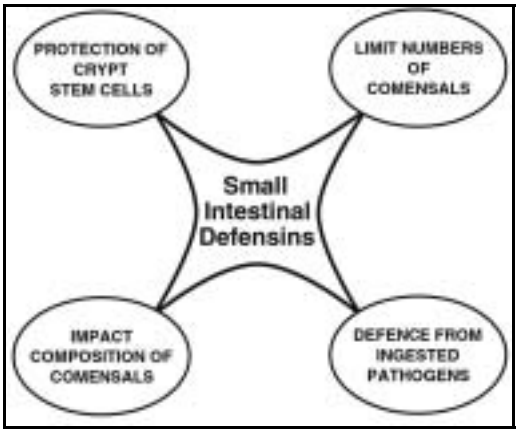

Figure 3: Four proposed functions of Paneth cell defensins.

CONCLUSIONS

Many lines of evidence support that Paneth cell defensins are key to innate immunity of the small bowel. These peptides may have additional physiologic roles. Paneth cells contribute actively to mucosal immunity by sensing bacteria and releasing microbicidal peptides at effective concentrations. The biosynthetic and processing pathways, the receptors for pattern recognition, and the signalling pathway(s) associated with apical secretion will require further study to understand this axis more completely. Given that microbial products stimulate Paneth cell secretion, it is possible that the mechanism of action of probiotic agents may, in part, involve modulating Paneth cell secretion.

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