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Antimicrobial Peptides as Defense at the Interface of the Host and Bacteria

Ulf Meyer-Hoffert

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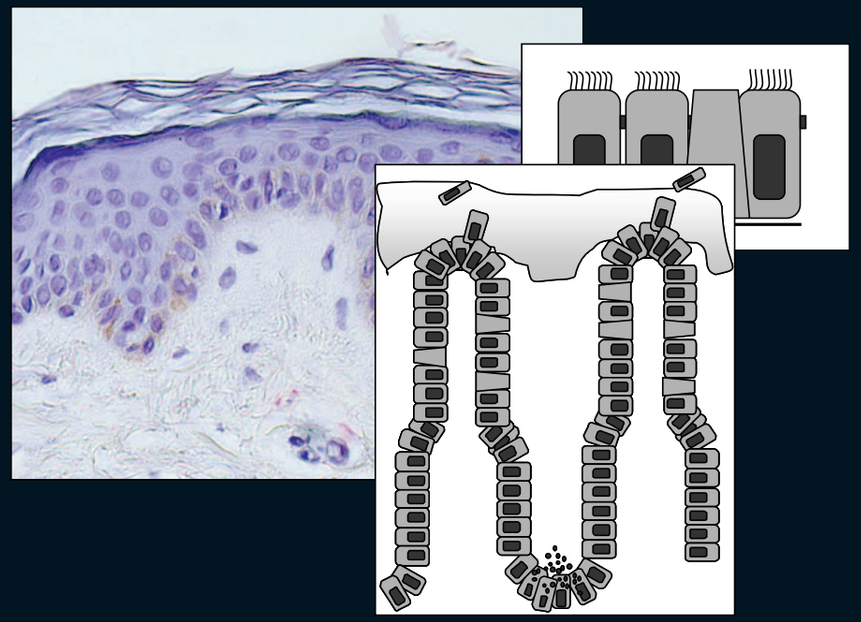

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„*Da steh ich nun, ich armer Tor,*
und bin so klug als wie zuvor.“

Faust I, Johann Wolfgang von Goethe

“pigmaei gigantum humeris impositi plusquam ipsi gigantes vident”

Isaac Newton

adopted from

“*nanos gigantium humeris insidentes*”

Bernard de Chartres

ABSTRACT

Microbes surround us. However, most of the time we are unaware of them. Our epithelial surfaces, which provide the physical barrier and separate us from the environment, are usually free of signs of a battle taking place. Over the past several years, it has been realized that the epithelium is capable of mounting its own battery of defensive chemicals. Besides the constituents of fluids, such as tears, saliva, and sweat that are secreted onto the surface of epithelia, antimicrobial peptides defend this barrier. The aim of this project was to investigate antimicrobial components at different body surfaces like lung, skin and intestine.

In the first study the human beta defensin-2 was discovered as the major antimicrobial peptide from lung epithelial cells against *P. aeruginosa*, a bacterium often affecting the lungs of patients suffering from cystic fibrosis. It was demonstrated that mucoid forms of *P. aeruginosa* as well as the proinflammatory cytokines IL-1beta and TNF-alpha are able to induce the expression human beta defensin-2 in respiratory epithelial cells.

In the second study the four human beta defensins hBD1-4 were examined in keratinocytes. The expression of three defensins, namely hBD2, -3 and -4 were inhibited by co-cultivation with retinoic acid. These defensins exhibited upregulation during keratinocyte differentiation and stimulation by pro-inflammatory cytokines and bacterial contact. Co-incubation with retinoic acid exhibited the induction of hBD2, -3 and -4. Since retinoic acid is used topically in certain skin diseases and leads sometimes to bacterial superinfection, the here described inhibitory effect of retinoic acid on the regulation of defensins could explain this phenomenon.

In the third study it was demonstrated that most of the antimicrobial components in the small intestine of mice localize to the mucus layer building an effective shield between the bacteria in the intestinal lumen and the epithelial cells. Mucus preparation exhibited broad antimicrobial activity. Besides alpha defensins, other antimicrobial active factors like lysozyme, phospholipase A2 and ribosomal proteins could be identified. The results of this study imply that the mucus is not only a physical barrier but serves as a structure to keep antimicrobial peptides and proteins in high concentration. Recent studies have suggested an important role of antimicrobial peptides and the mucus in inflammatory bowl diseases.

In the fourth study the heparin/heparan sulfate interacting protein (HIP), which is similar to the ribosomal protein L29, was identified as one of the major antimicrobial active substances in lung extracts by HPLC-fractionation, N-terminal sequencing and mass-spectrometry analysis. HIP/RPL29 was also detected in extracts of the small intestine. HIP/RPL29 exhibited broad antimicrobial activity and notably against *P. aeruginosa* strains. The HIP/RPL29 protein was found to be localized specifically to the epithelial surface of the lungs and intestines by immunohistochemistry. We suggest that HIP/RPL29 fulfils a function as an abundant antimicrobial factor of the epithelial innate defense shield against invading bacteria both in the lungs and in the small intestine.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Harder J, **Meyer-Hoffert U***, Teran LM*, Schwichtenberg L, Bartels J, Maune S, Schröder JM.
Mucoid *Pseudomonas aeruginosa*, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia.
Am J Respir Cell Mol Biol. 2000 Jun;22(6):714-21.

II. Harder J*, **Meyer-Hoffert U***, Wehkamp K, Schwichtenberg L, Schröder JM.
Differential gene induction of human beta-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid.
J Invest Dermatol. 2004 Sep;123(3):522-9.

III. **Meyer-Hoffert U***, Hornef MW*, Henriques-Normark B, Axelsson LG, Midtvedt T, Pütsep K, Andersson M
Enteric antimicrobial activity localizes to the mucus surface layer
Manuscript

IV. **Meyer-Hoffert U**, Hornef MW, Henriques-Normark B, Normark S, Andersson M, Pütsep K
Identification of Heparin/Heparan Sulfate Interacting Protein as a broad-spectrum antimicrobial protein in lung and small intestine
Manuscript

* authors contributed equally to the work

CONTENTS

LIST OF ABBREVIATIONS

AD	Atopic Dermatitis
AMP	Antimicrobial peptide
AP	Activator protein
CD	Cluster of difference
cDNA	Complementary DNA
CF	Cystic fibrosis
ELISA	Enzyme-linked immuno sorbent assay
FLG	Filaggrin
GAPDH	Glyceraldehye 3-phophate dehydrogenase
hBD	Human beta defensin
hCAP	Human cationic antimicrobial pro-peptide
HD	Human defensin
HIP	Heparin/heparan interacting protein
HNP	Human neutrophil peptide
HPLC	High performance liquid chromatography
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
mRNA	Messengar RNA
NF	Nuclear factor
PCR	Polymerase chain reaction
PLA_2	Phospholipase A_2
PMA	Phorbol-myristate-acetate
RA	Retinoic acid
RP	Reversed phase
RPL	Large ribosomal protein
RPS	Small ribosomal protein
RT	Reverse transcription
S.c.	Stratum corneum
TFA	Trifluoroacetic acid
Th	T helper cell
TNF-alpha	Tumor necrosis factor alpha

1 INTRODUCTION

1.1 BACTERIA - FOR BAD AND GOOD

We are living in a hostile world. Microbes surround us. There are bacteria, viruses, fungi and other infectious agents nearly everywhere – in the air, in our drinking water, even in our selves! The media reports of new threatening infectious agents in regular terms and paints the picture of a world where we better stay sterile or there will be no guarantee for a tomorrow. Is this really the truth?

Undoubtedly, there are numerous bacteria, viruses and fungi, which cause diseases and have been and still are a threat to mankind. Major medical inventions during the last century has improved life expectancy and quality like the discovery of antibiotics and the use of vaccination to fight and prevent infectious diseases. One might forget by looking at the numerous bacterial diseases that we are constantly exposed to bacteria – but are pretty healthy. We carry them on our skin, have them in the nose and throat and live fine with around 10^{18} bacteria in our gut – a number 10-times higher than the total number of our own body cells. This coexistence of bacteria and the host is quite stable, and we do not get inflammatory responses to these bacteria. One important reason is that the bacteria stay outside the body. They cover the epithelial lining but do not cross it. However, if those bacteria leave their niche and enter the body, they can cause disease. If for example the barrier of our skin is destroyed like in burnt patients, we are in acute danger to get bacteremia and life-threatening sepsis. This circumstance is obviously not the normal, "healthy", state and the aim of this thesis is to give a better understanding how the host protects itself from bacteria and remains in the status of keeping bacteria at a close but safe distance. Before getting *in medias res*, it is important to consider some general properties of immunity.

1.2 INNATE IMMUNITY – ANCIENT SYSTEM OF DEFENSE

When Elie Metchnikoff (also spelled Ilya Mechnikov) discovered the engulfment and destruction of bacteria by white blood cells at the end of the 19th century, the discovery of phagocytosis, which earned him the Nobel Prize in 1908 (Metchnikoff, 1908), modern immunity started.

The immune system protects organisms from infection with layered defenses of increasing specificity. Most simply, physical barriers prevent pathogens such as bacteria and viruses from entering the body. If a pathogen breaches these barriers, the immune system provides antimicrobial defense mechanisms. As a general paradigm, antimicrobial defenses can be divided into two systems: clonal ("acquired" or "adaptive") immunity and innate (nonacquired) immunity (table 1). The clonal immune

system uses B and T lymphocytes to mediate and amplify antigen-specific humoral and cellular responses. These responses require days to weeks for maximal activity, involve somatic gene rearrangement, and lead to immunologic memory. Although the acquired immune system represents the "crowning accomplishment of vertebrate immunity" (Fearon, 1997) and has been the focus of significant clinically related research (until now awarded with ten Nobel Prizes), evidence today suggests that the function of the acquired immune system is intimately tied to and complemented by the evolutionarily ancient but less highly glorified innate immune system (Janeway, Jr., 1989).

Innate immunity encompasses a complex of first-line host-defense elements. This host-defense system can provide recognition of microbial organisms as foreign (Mosser, 1994; Ulevitch & Tobias, 1994; Pearson, 1996; Medzhitov & Janeway, Jr., 1997b); secondly, incapacitation and elimination of pathogens (Boman, 1995); and thirdly, adjuvant magnification of the acquired immune response when such a response is warranted (Janeway, Jr., 1989; Fearon, 1997; Medzhitov & Janeway, Jr., 1997a). The elements of innate immunity do not function in isolation, but interact to ensure that the magnitude of the host response reflects the severity of the microbial threat.

Innate immune system	***Adaptive immune system***
Response is non-specific	Pathogen and antigen specific response
Exposure leads to immediate maximal response	Lag time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Found in nearly all forms of life	Found only in jawed vertebrates
Germ-line coded	Gene rearrangement
Perfect	Imperfect - autoimmunity

Table 1.

The main cells of innate immunity are macrophages and neutrophils - the phagocytes, which can be recruited to the place of inflammation - as well as epithelial cells, which are the outermost cells of the body. They build up the physical barrier and are capable of recognizing bacteria, eliminating them and recruiting specialized immune cells to the site of infection. Next, three specific epithelial linings are described in more detail.

1.3 EPITHELIAL CELLS – THE INTERFACE

A striking feature of evolution in the animal kingdom is the development of highly specialized epithelial surfaces. These interfaces of host-environment interaction provide vital physiologic functions including gas exchange, nutrient absorption, water conservation, and mechanical protection.

1.3.1 The Respiratory Epithelial Lining

The respiratory tract can be divided into the upper and lower respiratory tract. The function of the upper respiratory tract is besides the physical barrier function to moisture the air and to clean it from inhaled particles. The main function of the lower epithelium is the gas exchange.

The upper respiratory epithelium is more specifically described as a ciliated pseudostratified columnar epithelium due to the arrangement of the columnar epithelial cells; the nuclei are not aligned in the same plane and make it appear as though several layers of cells are present. In actuality, all cells make contact with the basement membrane and are therefore a single layer of cells, hence the epithelium is called pseudostratified. Goblet cells are present amongst the columnar cells and secrete a mucus, which keeps the epithelium moist and traps particulate material moving through the airway. The cilia of the respiratory epithelium beat in a concerted effort to move secreted mucus containing trapped foreign particles towards the oropharynx. This system keeps the lower respiratory tract mainly free of bacteria and prevents mucus accumulation in the lungs.

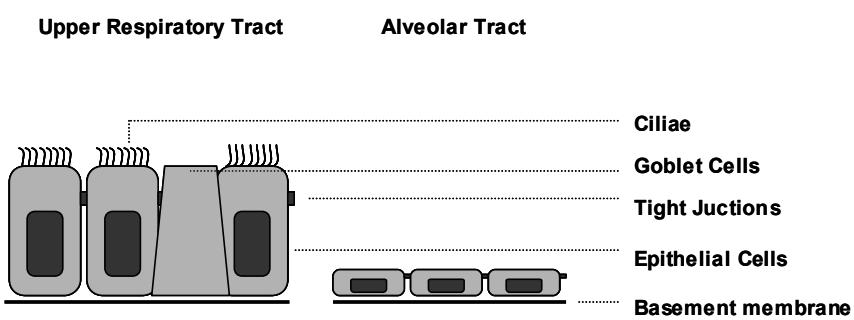

Figure 1. Schematic drawing of the respiratory epithelial lining at different anatomical localizations

The epithelial cells of the lower respiratory tract are called alveolar epithelial cells and are simple squamous epithelia. Goblet cells are absent. The cells produce surfactant, a phospholipid wetting agent, which upon release from the cell spreads along the alveolar epithelium thereby reducing surface tension and preventing collapse of the alveoli.

1.3.2 The Skin

Leaving the sea and expanding life on land was only possible for higher organisms by circumventing water loss of the body. This became the most important function of the epidermis, the epithelial lining of the skin. It is a stratified squamous, keratinized epithelium and can be divided into stratum basale, stratum spinosum, stratum granulosum and stratum corneum.

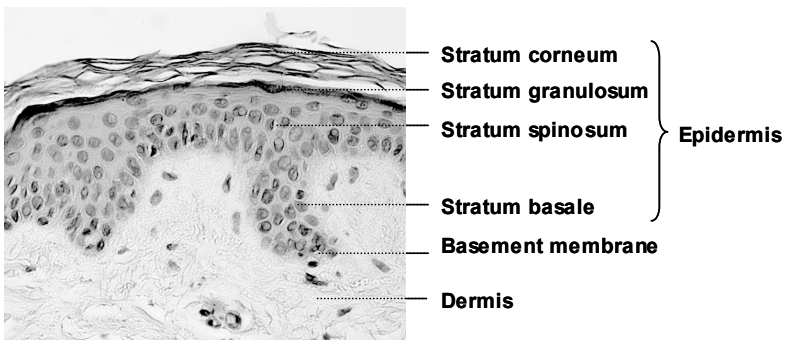

Figure 2. Histological picture of human skin showing the different epidermal layers of the cornifying epithelium

Healthy human skin is permanently in contact with the external environment and is covered with a characteristic microflora colonizing at the exposed surface and/or within the uppermost stratum corneum (S.c.) layers (Noble, 1989) but it is rarely infected. This is achieved by an intact physical barrier, which is formed by keratinocytes that have undergone metabolic and structural modifications during their terminal differentiation. Keratinocytes migrate from a proliferative basal layer towards the upper layers before ultimately ending with the demise of the keratinocytes via a specialized form of programmed cell death (Lippens *et al*, 2000) and transformation into corneocytes (Elias, 2007). The plasma membrane of corneocytes contains a lipid-rich and water-proof cornified cell envelope (Haftek *et al*, 1991; Roop, 1995; Steinert, 2000), which makes corneocytes very resistant against mechanical stress and protects the skin from water loss (Elias, 2005; Elias & Choi, 2005).

1.3.3 The Small Intestinal Epithelial Lining

The small intestine is the part of the gastrointestinal tract (gut) between the stomach and the large intestine and includes the duodenum, jejunum, and ileum. It is where the vast majority of digestion takes place. The intestinal wall folds in wrinkles called *plicae circulares*. From these project microscopic finger-like pieces of tissue called villi, which can be divided into the upper villus and the lower crypt region. The small

intestine is lined with a simple columnar epithelium. The epithelial cells have finger-like projections known as microvilli. The function of the *plicae circulares*, the villi and the microvilli is to increase the surface area available for secretion of enzymes and absorption of nutrients. Epithelial cells are shredded constantly at the top of the microvilli and new cells originate from the crypt region with a lifetime of around three days. The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. It is currently believed that four to six crypt stem cells reside at the +4 position immediately above the Paneth cells in the small intestine. Recent reports suggests that these stem cells are located at the crypt base (Barker *et al*, 2007b; Kayahara *et al*, 2003). Three differentiated cell types (enterocytes, goblet cells and enteroendocrine cells) form from transit-amplifying cells at the crypt–villus junction and continue their migration in coherent bands stretching along the crypt–villus axis. Goblet cells produce mucus, which covers the intestinal wall and is an integral part of the physical barrier. A unique repertoire of the small intestinal epithelial cells are the Paneth cells at the bottom of the crypts (Bry *et al*, 1994). They contain granules, which they release into the crypt lumen. Central components of these granules are antimicrobial peptides and proteins.

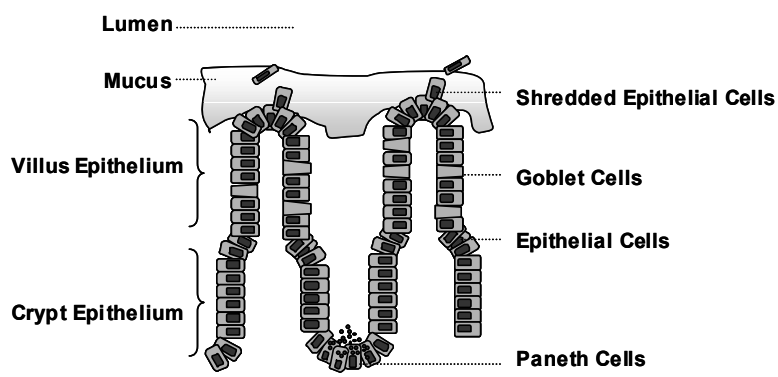

Figure 3. Schematic drawing of the small intestinal epithelial lining

The small intestine is colonized with bacteria with increasing numbers from the proximal to the distal region The absolute number of bacteria is rather stable under normal circumstances though sufficient nutrients are available. To some extent the mixed bacterial flora controls itself by competing with each other, but the host also provides an effective antimicrobial shield consisting of antimicrobial peptides and proteins, which will be discussed in the following section.

1.4 ANTIMICROBIAL PEPTIDES – ANCIENT WEAPONS OF HOST DEFENSE

Over the past three decades, it has been realized that the epithelium of all animals and plants is capable of mounting its own battery of defensive chemicals. Besides the constituents of fluids, such as tears, saliva, and sweat that are secreted onto the surface of epithelia, antimicrobial peptides (AMPs) defend this barrier.

1.4.1 Background

AMPs were fist characterized in 1981 by Håkan Steiner *et al*. (Steiner *et al*, 1981) nearly ten years after the same group demonstrated an inducible defense mechanism of the silk moth Hyalophora cecropia (Boman *et al*, 1972). This group of antimicrobial peptides was termed **cecropins** (Merrifield *et al*, 1994). New families of AMPs like **magains** (Zasloff, 1987) were discovered in the following years in all kind of life forms with an enormous diversity. The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animal, even those closely related. The reason for diversity might be that single mutations can dramatically alter the biological activity of each peptide, so the diversity probably reflects the species' adaptation to the unique microbial environments that characterize the niche occupied (reviewed in (Simmaco *et al*, 1998) and (Boman, 2000)).

The fundamental structural principle underlying all classes is the ability of the molecule to adopt a shape, in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule. This makes it possible for AMPs to interact with the negatively charged membrane of bacteria leading to their destruction. A model that explains the activity of most antimicrobial peptides is the Shai–Matsuzaki–Huang model (Matsuzaki, 1999; Yang *et al*, 2000; Shai, 1999). The model proposes the interaction of the peptide with the membrane, followed by displacement of lipids, alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell. The presence of cholesterol in the target membrane in general reduces the activity of antimicrobial peptides, due either to stabilization of the lipid bilayer or to interactions between cholesterol and the peptide (Matsuzaki, 1999). Similarly, it is believed that increasing ionic strength, which in general reduces the activity of most antimicrobial peptides, does so in part by weakening the electrostatic charge interactions required for the initial interaction.

1.4.2 Defensins

Defensins are one of the two most common types of AMPs. The term “defensin” was brought up by Robert Lehrer and co-workers (Ganz *et al*, 1985; Selsted *et al*, 1985) to term three short peptides with antimicrobial activity isolated from human neutrophil

granules. Defensins from vertebrates are small in size, are cationic, and have three intra-molecular disulfide bonds, mediated by six conserved cysteines (Bowdish *et al*, 2006). There are two main subfamilies: the α- and the β-defensins, based on the cysteine pairing and the length of the peptide fragments between the cysteines. All members of these two subfamilies, whose structure has been analyzed, show a similar, distinctive "defensin folding" composed of a predominant three-stranded β-sheet (Hill *et al*, 1991; Zimmermann *et al*, 1995; Szyk *et al*, 2006). The disulfide bonds in α-defensins are Cys1–Cys6, Cys2–Cys4 and Cys3–Cys5 (the number indicates the location of the Cys residue in the amino acid sequence from the N-terminus). They are expressed in human neutrophil cells, Paneth cells of the small intestine, and in a few epithelial cells. The four human α-defensins originally isolated from neutrophil cells are named as human neutrophil peptides (HNP)1–4; human defensin (HD)-5 and HD-6 are α-defensins produced by Paneth cells. The disulfide linkages in β-defensin are Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6. β-defensins are found in epithelial cells. Human β-defensins (hBD)-1–4 were originally isolated from human plasma (hBD-1, (Bensch *et al*, 1995)) and psoriatic scales (hBD-2 and hBD-3, (Harder *et al*, 1997; Harder *et al*, 2001)); hBD-4 has not yet been isolated, but identified solely by genomics (Garcia *et al*, 2001). The human genome suggests that there are at least 25 β-defensins that are yet to be discovered (Oppenheim *et al*, 2003). A third group of defensins, the θ-defensins, are only found in non-human primates, and are structurally unrelated to the α and β subfamilies (Tran *et al*, 2002; Cole *et al*, 2002).

Notably, the mouse shows certain differences to humans in respect to defensin expression und biology. First of all, mouse neutrophils do not express α-defensins (Eisenhauer & Lehrer, 1992). Mouse α-defensins from Paneth cells are called **cryptdins** and besides these the mouse Paneth cells express a repertoire of so-called cryptdin-related sequence peptides, which exhibit antimicrobial activity by dimer formation (Hornef *et al*, 2004). Mouse β-defensins have not been isolated yet. The genomic number outrages the human β-defensins by far (Hughes, 1999). mBD-1 and -3 have been shown to be expressed at certain epithelia and to be involved in bacterial defense (Bals *et al*, 1998a; Bals *et al*, 1999).

The functional importance of α-defensins in antibacterial protection has been demonstrated in knockout mice lacking the cryptdin-processing enzyme matrilysin (MMP7) and hence active peptides (Wilson *et al*, 1999). In contrast, mice engineered to produce an additional human defensin in the gut were less sensitive to *Salmonella* infection (Salzman *et al*, 2003). In humans, reduced levels of α-defensins were found in patients with Crohn's disease, (Wehkamp *et al*, 2004b; Wehkamp *et al*, 2005) suggesting a role for enteric α-defensins in the pathogenesis of inflammatory bowel disease.

Besides their antimicrobial activity defensin have been shown to possess cytokine-like function. They are able to act like chemokines (Yang *et al*, 1999; Yang *et al*, 2004) and have other cytokine-like functions, which suggested that their true function might be more like "alarmins" as truly antimicrobial agents, especially when concentrations are rather low and not sufficient to reach antimicrobial relevant levels (Oppenheim *et al*, 2007). In a recent report it was reported that a defensin mutation causes black coat color in domestic dogs (Candille *et al*, 2007).

1.4.3 Other Epithelial-derived Antimicrobial Peptides and Proteins

In addition to defensins, further epithelial-derived bactericidal peptides and proteins have been discovered.

The other main family of AMPs in mammals are the **cathelicidins**. Cathelicidins have a conserved proregion, cathelin, in common and a viable C-terminal antimicrobial domain. Upon activation, the C-terminal domain is cleaved off, liberating an active antimicrobial peptide (Zanetti, 2004). The human cationic antimicrobial pro-peptide, (hCAP)18 is the only human cathelicidin. It is cleaved to its active form LL-37. LL-37 was first identified in blood cells (Agerberth *et al*, 1989) but has been found at numerous locations in the body. Cathelicidin antimicrobial relevance in disease has been proposed in Kostmann's disease (Pütsep *et al*, 2002), rosacea (Yamasaki *et al*, 2007), *S. pyogenes* infection (Nizet *et al*, 2001) and Atopic Dermatitis (Ong *et al*, 2002).

The enzyme **lysozyme** was already discovered 1922 by Alexander Fleming (Fleming A, 1922). It damages bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. It is abundant in a number of secretions, such as tears, saliva, and mucus. Lysozyme is also present in the primary and secondary granules of the polymorphonuclear neutrophils.

A further antimicrobial enzyme expressed in Paneth cells and released into the intestinal lumen is **secretory phospholipase A_2** (Harwig *et al*, 1995), which is also secreted in the tear fluid (Qu & Lehrer, 1998).

Lactoferrin and **lipocalin** are antimicrobial proteins that act by their capacity to restrict the availability of iron, an essential microbial nutrient. Lactoferrin can damage microbes both by chelation of iron an by effecting membrane damage, the latter arising as a consequence of interactions mediated by amphipathic cationic sequences (Abrink *et al*, 2000). Lipocalin exhibits bacteriostatic activity against organisms that secrete siderophores in iron-limiting media and is inducible at epithelial sites (Goetz *et al*, 2002; Berger *et al*, 2006; Flo *et al*, 2004; Sørensen *et al*, 2003). Lipocalin-null mice are

more susceptible to systemic infection from organisms that synthesize siderophores (Berger *et al*, 2006; Flo *et al*, 2004).

The mechanism to restrict the availability of an essential ion, seems to be a common principle for some antimicrobial proteins. The advantages of such a mechanism are that it is very cost efficient for the host and very rapid in its action. **Psoriasin** has been found to be the main factor on human skin to protect from *E. coli* colonization most probably by the depletion of zinc (Gläser *et al*, 2005).

In a similar fashion like hBD-3 a further cationic protein was identified from healthy human skin – **RNase 7** (Harder & Schröder, 2002). RNase 7 exhibits antimicrobial activity independently from its RNase activity and belongs to the most abundantly expressed antimicrobial peptides and proteins in human skin (Harder & Schröder, 2005). In mice, it was shown that the RNase angiogenin 4 is involved in intestinal antimicrobial defense (Hooper *et al*, 2003). Moreover, antimicrobial activity has been reported for the epithelial-derived protease inhibitors **antileukoprotease**, also named secretory leukocyte protease inhibitor **SLPI**, (Wiedow *et al*, 1998) and **elafin** (Meyer-Hoffert *et al*, 2003).

During the identification of antimicrobial substances from tissue extracts, highly cationic factors have been found to have an antimicrobial activity. Among these were **histones** and **ribosomal proteins** (Tollin *et al*, 2003; Bergsson *et al*, 2005; Howell *et al*, 2003). When investigating antimicrobial peptides of the human small intestinal mucosa, fragments of histone 1 were found to have antimicrobial activity. Immunopositive staining for histone 1 was not only observed in the nucleus but also in the cytoplasm of some villous epithelial cells suggesting a non-nuclear function (Rose *et al*, 1998). In another relevant study, Hiemstra and colleagues isolated a H1 histone and found it to be a prominent antimicrobial component of interferon-gamma-stimulated mouse macrophage cells (Hiemstra *et al*, 1999). Ribosomal protein L30 was identified in human colon extracts as an antimicrobial protein (Howell *et al*, 2003). It shares N-terminal sequence similarity with RpL1, the antibacterial protein from *H. pylori* (Pütsep *et al*, 1999). It is tempting to speculate that antimicrobial domains of ribosomal proteins and histones were conserved during evolution and gained a function in antibacterial defense.

Recently, a new mechanism to generate antimicrobial active peptides has been proposed. Proteolytic cleavage of high molecular weight kininogen releases a peptide of high antimicrobial activity during systemic infection (Frick *et al*, 2006). This principle of innate immune defense was named "**the contact system**". Mainly, this is in concordance to the finding that proteolytically activated factors of the complement system exhibit direct antimicrobial activity (Nordahl *et al*, 2004). This mechanism is beneficial for the host since it provides an antimicrobial active substance in high

concentration within a very short time. It will be interesting to see in the future, whether such a proteolytic system is also available at the epithelial interface.

2 AIMS OF THE THESIS

The general aim of the work in this thesis was to identify major antimicrobial peptides and proteins at various epithelial linings and investigate their regulation and spatial localization.

The specific aims in the individual papers were:

I. To identify a major lung epithelial-derived antimicrobial factor acting against *Pseudomonas aeruginosa* and to study its translational regulation in cultured airway epithelial cells.

II. To study the expression and regulation of the four human beta defensin hBD-1-4 in cultured keratinocytes and to investigate whether their expression depends on keratinocyte differentiation. Specifically to analyze whether retinoic acid influences beta defensin expression in keratinocytes.

III. To analyze the antimicrobial active compounds in mucus preparations from mouse small intestine and to compare those to crypt and whole intestinal preparations.

IV. To identify antimicrobial active proteins/peptides from mouse lung tissue and to compare the findings to the situation in the small intestine.

3 METHODOLOGY

This chapter describes the principles of the methods that are central to the work presented in this thesis and discusses their advantages and limitations. For more information about these and the other methods used, please refer to the specific papers.

3.1 EXTRACTION OF BIOLOGICAL MATERIAL

Two different methods were used to prepare peptide extracts from biological material. In the first paper, the supernatant of cultured airway epithelial cells was collected, diafiltered to reduce the volume and subsequently used for High performance liquid chromatography (HPLC) separation. The advantage of this method is that only few material gets lost, especially peptides and other molecules with a size lower than 3 kDa, which was the cutoff level of the filtration system. Since only the supernatant was collected, mainly secreted peptides/proteins were analyzed. However, proteins like HIP/RPL29 (paper IV), which localizes to the outer membrane might be missed. This could be the reason, why HIP/RPL29 was not detected as a *Pseudomonas aeruginosa*-killing antimicrobial protein from A549 cells in paper I.

In paper III and IV, tissues from the organ of interest were removed, and immediately frozen in liquid nitrogen in order to avoid action of endogenous proteolytic processes. Frozen tissues were ground into small pieces in a mortar and dissolved in 60% acetonitrile with 1% trifluoroacetic acid (TFA), frozen at –80°C and lyophilized. The remaining pellet was redissolved using 10% ethanol. To further analyze the material, HPLC was used to separate compounds by hydrophobicity.

3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC allows separation of complex mixtures by different biochemical properties. These depend on the column used for separation. In paper I a *P. aeruginosa* affinity column was used. The *P. aeruginosa* affinity column was prepared using an N-hydroxy-succinimide (NHS)–activated sepharose column. Subsequently, 5 ml of mucoid *P. aeruginosa* suspension (10^9 bacteria/ml) (clone PA-O) were added to this preactivated column with a flow rate of 1 ml/min. Application of the bacteria to the column was performed five times to increase the coupling yield of the bacteria. The column was deactivated by washing three times with 0.5 M aqueous ethanolamine containing 0.5 M NaCl, pH 8.3, followed by 0.1 M sodium acetate containing 0.5 M NaCl, pH 4. Subsequently, the cell culture supernatant was applied to the affinity column that had been previously equilibrated with 10 mM phosphate buffer, pH 7.4. The eluate was concentrated using Amicon filters and diafiltered against 0.1%

trifluoroacetic acid (TFA) for subsequent reversed phase (RP) HPLC. This strategy was chosen, since analyzes of complex mixtures of biological material can become rather challenging, especially when some high-tech techniques like mass spectrometry facilities are not available, which was the case in our lab during the time of paper I. The strategy was based on the assumption that *Pseudomonas*-killing peptides interact directly with the bacteria. Other putative *Pseudomonas*-killing factors, which act for example by ion depletion, are not necessarily detected by this method.

In paper III and IV, unlike in paper I, extracted compounds were administered directly to a reversed phase column. The name "reversed phase" has a historical background. In the 1970s most liquid chromatography was done on non-modified silica or alumina with a hydrophilic surface chemistry and a stronger affinity for polar compounds - hence it was considered "normal". The introduction of alkyl chains bonded covalently to the support surface reversed the elution order (Molnar & Horvath, 1976). Now polar compounds are eluted first while non-polar compounds are retained - hence "reversed phase". All of the mathematical and experimental considerations used in other chromatographic methods apply (i.e. separation resolution proportional to the column length). To achieve an acceptable separation of substances in our studies, a slow gradient increase was necessary resulting in a three hour separation time compared to 45 minutes in paper I. Advantageous of this strategy was that various separation techniques were omitted allowing the identification and biological characterization of substances derived from one single mouse lung and intestine.

3.3 ANTIMICROBIAL ASSAYS

In most of the investigations in this work a thin ager plate assay was used as described in Hultmark *et al* (Hultmark *et al*, 1983), where fractions to be investigated are pipetted into wells of an agarose plate. They diffuse into the ager and can interact with the bacteria, which have been diluted into the ager before. After one hour incubation, a second layer of Luria Bertani (LB) medium is pipetted onto the plate to supply nutrients for the bacteria. After an overnight incubation the zone diameters are measured. The inhibition zone assay is a quick and informative method to analyze the total antimicrobial activity in peptide/protein extracts and chromatographic fractions. It has got its drawbacks if it comes to compare different extracts or synthetic peptides against each other in killing efficiency.

For that purpose, the colony forming unit (CFU) assay was chosen. This assay was performed in microtiter plates, where serial dilutions of the peptide were mixed with bacteria. The mixture was incubated for one hour and aliquots were plated on agar plates. Next day, the number of surviving colonies was counted.

In paper III we used in addition a method to visualize killing by so-called life-dead staining of bacteria. Live bacteria were distinguished from dead by the life-dead kit (Molecular Probes Inc.). The kit comprises two probes: calcein AM and ethidium homodimer-1. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product; thus, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Ethidium homodimer-1 is a high-affinity, red-fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. The advantage of this method is that overnight incubation is not needed and the result of the experiment can be seen straight forward.

3.4 TRANSCRIPTIONAL REGULATION

One way of analyzing the expression of peptides or proteins is to study the levels of their mRNA. This can be performed using polymerase chain reaction (PCR) based methods. In RT-PCR, RNA, which is unstable, is isolated and reversely transcribed into cDNA, which is very stable and may be stored for years in the freezer. Amplification of DNA by the polymerase chain reaction was a breakthrough in molecular biology, honored with the Noble Prize in 1993 (Mullis & Faloona, 1987; Bartlett & Stirling, 2003). Specific primers are used and directed to a defined part of the cDNA, which is amplified during repeated changes of the reaction temperature in the presence of the enzyme polymerase. The PCR products are loaded in gels, where the size of the amplified product is confirmed. Non-specific amplification is common. Therefore, confirmation of the correct sequence by sequencing should be performed. RT-PCR is a good method to detect the transcription of a particular gene. However, it is not a good method for quantitative analysis. The problem is related to the saturation effect, which occurs late in PCR amplification. Saturation is achieved when the nucleotides for the new DNA strands are consumed and the amount of synthesized DNA reaches a plateau. This can be prevented by lowering the numbers of amplification cycles and by comparing the PCR bands with defined bands of an internal standard. The internal standard should be a transcript that is not affected by the experimental conditions. In paper I we have chosen the housekeeping gene glyceraldehyde 3-phophate dehydrogenase (GAPDH). This method allows the semi-quantitative analysis of a PCR product but still the accuracy of analysis is rather low.

This problem has been partly solved in real time PCR, which was used in paper II. This technique continuously monitors the cycle-by-cycle accumulation of the fluorescently labeled PCR product. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using the software of the manufacturer (LightCycler Software, Roche Molecular Biochemicals). The software first normalizes each sample by detecting the background fluorescence present in the initial cycles. Then a fluorescence threshold at 5% of full scale is set, and the software determines the cycle number at which each

sample reaches this threshold. This threshold fluorescence cycle number correlates inversely to the log of the initial template concentration. Relative transcript levels were corrected by normalization based on the GAPDH transcript levels. The specificity of the amplification products was further verified by subjecting the amplification products to electrophoresis on a 2% agarose gel.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Induction of an Antimicrobial Peptide

The strategy of paper I, published in 2000, was based on studies, which revealed that apart from skin, normal human airway epithelia express mRNA for the inducible hBD-2 (Harder *et al*, 1997; Bals *et al*, 1998b), which is strongly upregulated in the respiratory epithelia of patients with inflammatory lung diseases and cystic fibrosis (Singh *et al*, 1998). We hypothesized that human airway epithelial cells express upon stimulation an antimicrobial active factor, which interacts with the bacterial membrane. Therefore, we purified antimicrobial activity from supernatants of the A549 lung epithelial cell line, previously stimulated with *P. aeruginosa*, by subsequent high performance liquid chromatography using a *P. aeruginosa* affinity column. NH2-terminal sequencing of a major bactericidal compound revealed it to be identical with hBD-2. A mucoid phenotype of *P. aeruginosa* (Fig. 4), but not two nonmucoid *P. aeruginosa* strains, high concentrations (> 10 µg/ml) of *P. aeruginosa* lipopolysaccharide, tumor necrosis factor alpha, and interleukin (IL)-1beta, but not IL-6, dose-dependently induced hBD-2 mRNA in cultured normal bronchial, tracheal, as well as normal and CF-derived nasal epithelial cells. Genomic analysis of hBD-2 revealed a promoter region containing several putative transcription factor binding sites, including nuclear factor (NF) kappa B, activator protein (AP)-1, AP-2, and NF-IL-6, known to be involved in the regulation of inflammatory responses. Thus, hBD-2 represents a major inducible antimicrobial factor released by airway epithelial cells either on contact with mucoid *P. aeruginosa* or by endogenously produced primary cytokines. Therefore, it might be important in lung infections caused by mucoid *P. aeruginosa*, including those seen in patients with CF.

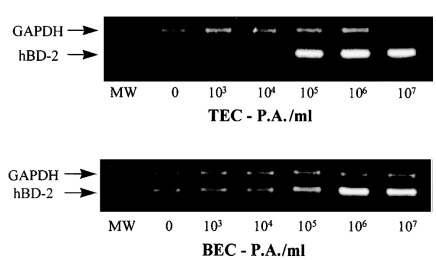

Figure 4.
Heat-killed mucoid *P. aeruginosa* (P.A.) upregulates mRNA expression of hBD-2 in airway epithelial cells. Analysis of gene expression of hBD-2 in cultured primary tracheal (TEC) and bronchial (BEC) epithelial cells using RT-PCR. Migration position of hBD-2 (255 bp) and GAPDH (360 bp) specific amplification products are indicated. A 100-bp ladder was used as molecular weight (MW) size marker.
Am J Respir Cell Mol Biol. 2000 Jun;22(6):714-21

Relevance for antimicrobial peptides in CF has been proposed due to the following hypothesis. Antimicrobial peptides are secreted into a micrometer-thick mucus layer directly overlying the epithelium. The concentration and composition of electrolytes

within this antimicrobial-rich unstirred layer are regulated by a variety of pumps and channels, which maintain conditions that maximize the antimicrobial activity of these defenses. In CF, this homeostasis is disturbed by the genetic defect in the cystic fibrosis transmembrane-conductance regulator. It has been suggested that antimicrobial peptide defenses fail to provide an effective barrier resulting in bacterial overgrowth, especially of *P. aeruginosa* and chronic, tissue-destructive inflammation (Smith *et al*, 1996; Goldman *et al*, 1997).

4.2 PAPER II

Inhibition of Antimicrobial Peptide Production

Human skin is able to mount a fast response against invading harmful bacteria through the rapid production of inducible peptide antibiotics such as the human beta-defensins. To gain more insight into the role and regulation of inducible beta-defensins in the innate immunity of human skin, we investigated whether gene induction of the human beta-defensins hBD-1, -2, -3, and -4 in keratinocytes is regulated in a similar manner. Therefore, we performed a comparative study of gene expression of these four hBD in primary cultured keratinocytes using real-time PCR. A basal mRNA expression was observed for all four hBDs in cultured primary keratinocytes, which strongly increased for hBD-2, -3, and -4 during Ca^{2+}-induced differentiation of the keratinocytes. This effect was completely abolished when the keratinocytes were pre-treated with all-trans-retinoic acid (RA). Furthermore, the differential induction of hBD-2, -3, and -4 gene expression in keratinocytes by proinflammatory cytokines, phorbol-myristate-acetate (PMA), and bacteria was inhibited by more than 90% when the keratinocytes were pre-incubated with RA. Inhibition of IL-1beta-mediated hBD-2 induction through RA was further confirmed by gene reporter assays (Fig. 5) and western-blot analysis. We conclude that RA is a potent inhibitor of beta-defensin induction in keratinocytes and might downregulate the inducible innate chemical defense system of human skin.

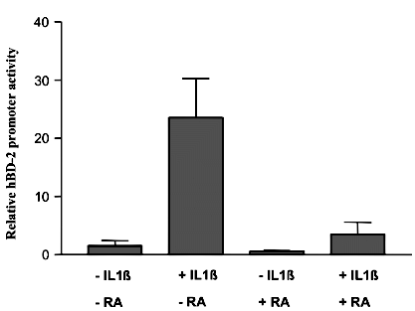

Figure 5.
Primary keratinocytes were transiently transfected with a luciferase gene reporter vector containing hBD-2 promoter. Twenty-four hours after transfection cells were stimulated with 10 ng per ml IL-1beta for 16 h. Stimulations were performed either without addition of 10^{-7} M all-trans-RA (-RA) or with simultaneous addition of 10^{-7} M all-trans-RA and an additional pre-incubation for 24 h with 10^{-7} M all-trans-RA (+RA). After stimulation cells were harvested and hBD-2 promoter activity was determined as a ratio between firefly and renilla luciferase activity. The data represent the mean ±SD of triplicate cultures.
J Invest Dermatol. 2004 Sep;123(3):522-9.

This finding might explain the observed overgrowth by *Staphylococcus. aureus* when retinoic acid is used as a topical treatment in dermatology (Graham *et al*, 1986; Leyden

& James, 1987). Inhibition of antimicrobial peptides was proposed as a concept is some bacterial diseases and might be a relevant pathogenic factor in bacterial infection. It was shown that *Neisseria gonorrhoeae* downregulates expression of the human antimicrobial peptide LL-37 (Bergman *et al*, 2005). Suppression of NF-kappaB-mediated beta-defensin gene expression in the mammalian airway by the *Bordetella* type III secretion system was reported (Legarda *et al*, 2005). Furthermore, it was shown that early in *Shigella spp*. infections, expression of the antibacterial peptides LL-37 and human beta-defensin-1 was reduced or turned off (Islam *et al*, 2001). The downregulation was detected in biopsies from patients with bacillary dysentery and in *Shigella*-infected cell cultures of epithelial and monocyte origin. Inducing the body's own antimicrobial peptides might be a therapeutic option in the future.

4.3 PAPER III

Localization of Antimicrobial Peptides and Proteins

The intestinal mucosa is constantly exposed to a dense and highly dynamic microbial flora and challenged by a variety of enteropathogenic bacteria. Antibacterial protection is provided, in part by Paneth cell-derived antibacterial peptides such as the α-defensins. The mechanism of peptide-mediated antibacterial control and its functional importance for gut homeostasis has recently been appreciated in patients with Crohn's diseases localized to the ileum. In the third paper we analyzed the spatial distribution of antimicrobial peptides within the small intestinal anatomical compartments such as the intestinal crypts, the overlaying mucus and the luminal content. Preparations from the different intestinal locations as well as whole mouse small intestine were extracted and separated by RP-HPLC. Antibacterial activity was determined in extracts, and the presence of antimicrobial peptides/proteins was confirmed by N-terminal sequencing, mass spectrometry analysis and immunodetection.

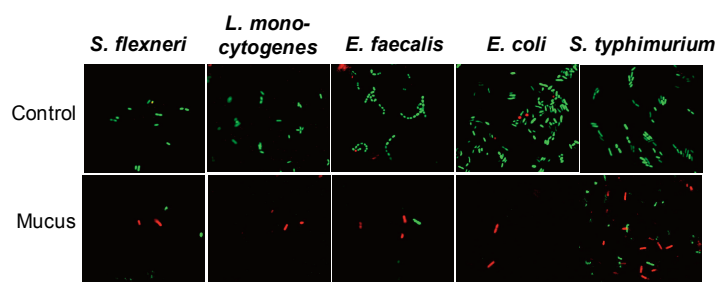

Figure 6
Life-dead staining of commensal and pathogenic bacteria associated with mucus. The viability of bacteria in close contact with isolated mucus (lower part, mucus) or control matrix (upper part, control matrix) was determined by life-dead staining and microscopy after 1 h incubation. Live bacteria are shown in green and dead bacteria in red.

The secreted antibacterial activity was largely confined to the mucus layer, whereas only minute activity was noted in the luminal content. A spectrum of antimicrobial peptides was identified in isolated mucus, which exhibited strong and contact-dependent antibacterial activity against both commensal as well as pathogenic bacteria. These findings show that secreted antimicrobial peptides are retained by the surface-overlaying mucus and thereby provide a combined physical and antibacterial barrier to prevent bacterial attachment and invasion. This distribution facilitates high local peptide concentration on vulnerable mucosal surfaces, while still allowing the presence of an enteric microbiota – or to say in simple words: antimicrobial peptides ain't floatin' 'round. On the first view this finding might appear to be quite trivial but it is of fundamental importance since it guarantees that antimicrobial activity is kept at high local concentration, which circumvents adaptation of the microflora in the form of antimicrobial resistance. This could occur when a gradient of antimicrobial components would arise throughout the intestine. Furthermore, the complex mixture of active peptides and proteins, in total we identified fifteen different antibacterial factors present in non-inflamed small intestinal preparations, with different antibacterial mechanisms prevents antimicrobial resistance, too. Our finding are relevant for the intestinal bacterial homeostasis and Crohn's disease. The mucus barrier in patients suffering from Crohn's disease seem to be distorted leading to direct contact of commensal bacteria to the epithelial lining, which was not the case in healthy individuals (Swidsinski *et al*, 2005b; Swidsinski *et al*, 2005a). Furthermore, certain patients with Crohn´s disease ileitis have reduced α-defensin levels (Wehkamp *et al*, 2004b; Wehkamp *et al*, 2005).

4.4 PAPER IV

A new Player in the Game

The lungs are continuously exposed to a broad array of microbes through inhalation and microorganisms that escape clearance by the upper airway mucociliary motion will deposit in the alveolar compartment of the lower airways. The pulmonary epithelium in the alveolar compartment is covered by a thin aqueous layer that contains surfactant proteins but also microbicidal components. We have here identified the epithelial cell-surface-expressed heparin/heparan interacting protein (HIP/RPL29) by HPLC-fractionation, N-terminal sequencing and mass-spectrometry analysis as a major antimicrobial component in extracts of mouse lung tissue. HIP/RPL29 was furthermore detected in extracts of mouse small intestinal tissue. Mouse and human HIP/RPL29 exhibited broad antibacterial activity and notably against *P. aeruginosa* strains. The HIP/RPL29 protein was demonstrated to be localized to the epithelial cells and cell surface of the lungs and intestines by immunohistochemistry. We suggest that HIP/RPL29 fulfils a function as an abundant antibacterial factor of the epithelial innate defense shield against invading bacteria both in lungs and in the small intestine.

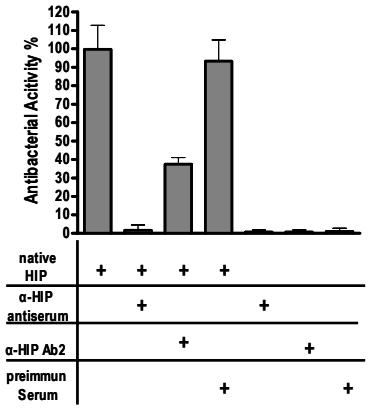

Figure 7.
Inhibition of bactericidal activity against *S. pyogenes* by coincubation of the HIP/RPL29 containing pooled HPLC-fractions with anti-HIP/RPL29 antiserum, a second anti-HIP/RPL29 antibody (Ab2) as well as the preimmunisation serum. Killing of bacteria was tested using the diffusion assay. Antibacterial activity of 1 µl of HIP/RPL29-containing fraction was set as 100%, bars represent mean of n=3 and error bars display standard deviation.

HIP/RPL29 was first described as a highly basic (calculated pI=11.66) heparin/HS-interacting protein HIP in uterine endometrium with strong cell surface expression in luminal uterine epithelial cells (Rohde *et al*, 1996). Significant expression of HIP/RPL29 was reported in murine embryonic tissues, notably the eye, liver, lung, and cartilage of the developing bones (Kirn-Safran *et al*, 2000) and predominantly localized to epithelial cells. By homology search it was found that HIP/RPL29 is identical to the cDNA sequence of ribosomal protein L29 (Law *et al*, 1996). Although the exact *in vivo* function of HIP/RPL29 is not known, remarkably diverse biological properties have been attributed to HIP/RPL29. It is suggestive that HIP/RPL29 is not produced to the same extent and tissue range in adult individuals as during fetal life supporting the idea that HIP/RPL29 function is not associated exclusively with protein synthesis. It is not unusual for ribosomal proteins to have an extra-ribosomal function (Wool *et al*, 1995; Fujita *et al*, 1994; Zimmermann, 2003) and the presence of HIP/RPL29 at the cellular surface is not unique since another ribosomal protein (LBP/p40/RPSa) also was found to be exposed at the cell surface (Landowski *et al*, 1995). Notably, knock-out mice lacking HIP/RPL29 are viable but exhibit growth deficiencies during fetal development (Kirn-Safran *et al*, 2007). The idea that HIP/RPL29 plays more than one role in cellular biology is supported by described *in vivo* and *in vitro* changes in the cytologic distribution of HIP/RPL29 during mouse mammary epithelial cell expression and differentiation (Kirn-Safran *et al*, 2002).

Ribosomal proteins have an affinity for negatively charged molecules and it is possible that motifs with basic amino acids of ribosomal proteins were conserved and became part of the innate immune defence. *Helicobacter pylori* grown *in vitro* displayed antibacterial activity that could be traced to several cecropin-like peptides derived from the N-terminal part of ribosomal protein L1 (Pütsep *et al*, 1999). Moreover, ribosomal proteins were identified as antimicrobial agents in the skin mucus of fish (Bergsson *et*

al, 2005) and the ribosomal proteins RPL30, RPL39 and RPS19 were detected by their antimicrobial activity in human colon epithelium (Howell *et al*, 2003) and human colon (Tollin *et al*, 2003). Ubiquicidin, which is a bactericidal protein that can be isolated from the cytoplasm of IFN-gamma activated macrophages is identical to RPS30 (Hiemstra *et al*, 1999). Taken together all these data illustrate that some ribosomal proteins might have gained a role in immunity as antimicrobial agents.

5 CONCLUDING REMARKS

5.1 THE MAJOR FINDINGS

- hBD-2 is an - likely the most important - inducible antimicrobial peptide released by human airway epithelial cells against *P. aeruginosa.*

- hBD-2, -3 and -4 but not hBD-1 are inducible antimicrobial peptides in keratinocytes. Their expression are enhanced during keratinocyte differentiation. Retinoic acid inhibits the expression and induction of hBD-2, -3 and -4.

- The vast majority of antimicrobial peptides and proteins expressed in the small intestine localizes to the mucus layer, which is an active antibacterial barrier.

- HIP/RPL29 is an abundant antibacterial factor of the epithelial innate defense shield against invading bacteria both in lungs and in the small intestine.

5.2 FUTURE PERSPECTIVES – PERSONAL VIEW

So, where are we now? As Dr. Faust[1] in desperation of his lack of knowledge, though he has studied philosophy, law, medicine and "unfortunately" theology – or can we see the bright future from the shoulders of the giants we are standing on?

Insight into the innate barrier shield of AMPs, mucus, and structural proteins are changing the way how we look upon certain diseases. For example, Crohn's disease has been considered to be exclusively a T-cell mediated inflammatory disease. Recent findings suggest that defensin-deficiency, in some cohorts due to a mutation in an intracellular receptor, NOD-2, leads to inflammation by the body's own microflora (reviewed in (Wehkamp *et al*, 2007)). This new pathophysiological concept might lead to new therapeutic strategies in the future. Giving a probiotic bacterium, *E. coli* Nissle 1917, instead of anti-inflammatory drugs or antibiotics as a treatment to Crohn's patients is a first step into this direction. The effect by *E. coli* Nissle 1917 might be due to its ability to stimulate defensin expression in intestinal epithelial cells (Wehkamp *et al*, 2004a). The advantage of such a system is that the antimicrobial peptides are endogenously produced in the natural location. Applying antimicrobial peptides orally might be of lower therapeutic potential considering the findings in paper III showing that antimicrobial peptides are not normally present in the intestinal lumen. The microflora of the gut would react against orally administered antimicrobial peptides like it does to other antibiotics – it will adept by changing to another stable state – probably with some diarrhea in between, and there will be the risk that AMP resistant bacteria are selected, which can subsequently be transmitted to other individuals. Anyway, time will tell since already today HD-5 engineered plants are being raised with the purpose to be given to Crohn's patients (Wehkamp, personal communication).

Furthermore, the concept of the dermatologic disease atopic dermatitis (AD) is being renewed, which was thought to be a Th2-driven and now is thought to arise from a barrier defect. The crucial role of the epidermal barrier homeostasis is demonstrated by mutations in the filaggrin (FLG) gene. Filaggrin is part of the cornified cell envelope in the epidermis and belongs to the family of S100 fused proteins. Recently, null mutations in the FLG gene were proven to underlie ichthyosis vulgaris (Smith *et al*, 2006) (Barker *et al*, 2007a) and determine major susceptibility to early onset AD (Barker *et al*, 2007a) (Palmer *et al*, 2006). These mutations. cause a complete loss of processed filaggrin products, leading to an impaired physical barrier and to higher infection rates in affected skin (Smith *et al*, 2006). Thus, skin barrier dysfunction is today suggested to play an important role in the development of AD and its associated diseases (Irvine, 2007).

[1] „Faust I", 1808 – Johann Wolfgang von Goethe, *1749 - †1832

Bacterial resistance against conventional antibiotics is an emerging health problem making the development of novel antibiotics a highly necessary issue (Normark & Normark, 2002). AMPs, both in humans and in other species, may be used as antibiotics *per se*, or serve as motives for customly designed peptides. However, peptides are not the optimal drug, as they are expensive to produce and difficult to deliver. Moreover, AMPs can become cytotoxic at certain concentrations (Wencker & Brantly, 2005). A preferable way would be to induce the expression of endogenous AMPs by drug-like compounds. The advantage would be that the antibiotics would be produced at their natural localization, which might reduce unwanted side effects like the cytokine-like function of AMPs leading to inflammation. It will be challenging to induce AMPs without inducing proinflammatory cytokines at the same time and by this strengthen the epithelial barrier without recruiting inflammatory cells. Overexpression of AMPs might also become dangerous as shown for the mBD-6, which induced muscle degeneration in mice (Yamaguchi *et al*, 2007).

It seems that the practical use of AMPs is something we have to wait for. However, the knowledge of these innate immune factors has changed the way we think about infection and the healthy state.

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