From the Department of Medical Biochemistry and Biophysics,
Karolinska Institutet, Stockholm, Sweden

Antimicrobial peptides and pathogenic Neisseria

Experimental studies in mouse, man and rat

Peter Bergman

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“The truth is out there…“

Agent Mulder, The X-files

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To Linda, Wilhelm and Emilia

Abstract

Antimicrobial peptides are important effector molecules of innate immunity. In this thesis, the focus is on antimicrobial peptides of the cathelicidin family, i.e. LL-37 in man, CRAMP in mouse and rCRAMP in rat. Expression of these cathelicidins in tissues and cells has been analyzed, and their functional relevance has been studied in relation to the human bacterial pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis.*

A peptide/protein extract was made from human colon mucosa and several antimicrobial peptides and proteins were identified by HPLC purification. We propose that this complex mixture of antimicrobial peptides and proteins provides a functional barrier protecting the human colon against invading microorganisms. One of the identified peptides, the human cathelicidin LL-37, is also expressed in cervical epithelial cells. Infection of these cells with *Neisseria gonorrhoeae* (gonococci) resulted in down-regulation of LL-37. Further, this peptide exhibited potent bactericidal activity against *Neisseria gonorrhoea*, suggesting that down-regulation of LL-37 may facilitate invasion of gonococci in the female genital tract.

The brain – on the other hand – is rarely infected, and the protective mechanisms remain to be fully elucidated. A peptide/protein extract of rat brain was found to be active against bacteria. Depletion experiments showed that the cathelicidin rCRAMP accounted for a large portion of this activity. Using RT-PCR and Western blot analysis, rCRAMP was localized to distinct regions of the brain. In addition, rCRAMP was found to be a potent killer of the neuropathogenic bacterium *Neisseria meningitidis*.

Meningococcal infection was studied in mice expressing the human complement regulator CD46. These mice were found to be highly susceptible to meningococcal infection. After meningococcal challenge, bacteria were found in cerebrospinal fluid of CD46 mice, but not in control mice, demonstrating that CD46 is crucial for establishing meningococcal infection. Finally, the role of mouse CRAMP in meningococcal infection was investigated. By immunohistochemistry, CRAMP was detected in the blood brain barrier and meninges after infection. CRAMP-KO mice were used to evaluate the role of CRAMP *in vivo*. Bacterial crossing of the blood brain barrier occurred both in CRAMP-KO mice and in control mice. However, CRAMP-KO mice exhibited higher bacterial counts in blood, liver and spleen six hours post infection, demonstrating a non-redundant and early effect of CRAMP in meningococcal sepsis.

Considering the emerging bacterial resistance against conventional antibiotics, it is important to investigate novel ways of treating infections. Isolation of antimicrobial peptides from tissue extracts and a detailed understanding of their regulation, may lead to the development of novel strategies in the treatment of infectious diseases.

List of publications

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I: Tollin M., **Bergman P.**, Svenberg T., Jörnvall H., Gudmundsson G.H. and Agerberth B. (2003). Antimicrobial peptides in the first line defence of human colon mucosa. *Peptides* 24: 523-530

II: **Bergman P.***, Johansson L.*, Asp V., Plant L., Gudmundsson G.H., Jonsson AB. and Agerberth, B. (2005), Neisseria gonorrhoeae down-regulates the expression of the human antimicrobial peptide LL-37. *Cellular Microbiology*, 7: 1009-1017

III: **Bergman P.**, Termén S., Johansson L., Nyström L., Arenas E., Jonsson AB., Hökfelt T., Gudmundsson G.H., and Agerberth B. (2005) The antimicrobial peptide rCRAMP is present in the central nervous system of the rat. *Journal of Neurochemistry* 93: 1132-1140

IV: Johansson L., Rytkönen A., **Bergman P.**, Albiger B., Källström H., Hökfelt T., Agerberth B., Cattaneo R. and Jonsson AB. (2003) CD46 in meningococcal disease. *Science* 301: 373-375.

V: **Bergman P.**, Johansson L., Wan H., Gallo R.L., Gudmundsson G.H., Hökfelt T., Jonsson AB. and Agerberth B. (2005) Induction of the antimicrobial peptide mouse CRAMP in the blood brain barrier and meninges after meningococcal infection. *Submitted.*

* The first and second authors contributed equally to this work

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Abbreviations

AD	atopic dermatitis
AMP	antimicrobial peptide
ASF	airway surface fluid
BBB	blood brain barrier
BPI	bactericidal permeability-increasing protein
CAF	CD8(+) cell antiviral factor
CAMP	cathelicidin antimicrobial peptide
CD	cluster of differentiation
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CF	cystic fibrosis
CGD	chronic granulomatous disease
CNS	central nervous system
CRAMP	cathelicidin related antimicrobial peptide
CVO	circumventricular organs
ECP	eosinopilic cationic protein
ELISA	enzyme-linked immuno sorbent assay
FPR	formyl peptide receptor
FPRL	formyl peptide receptor like
GAS	group A Streptococcus
GBS	group B Streptococcus
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IGF-1	insulin-like growth factor 1
IκB	inhibitory kappa B
IL-1β	interleukin-1β
KO	knock-out
LBP	lipopolysaccaride binding protein
LOS	lipooligosaccaride
LPS	lipopolysaccaride
MARCO	macrophage receptor with collagenous structure
MBL	mannose binding lectin
MDDC	monocyte derived dendritic cells
MHC	major histocompatibility complex
mRNA	messenger RNA
MyD88	myeloid differentiation factor 88

NFκB	nuclear factor kappa B
NO	nitric oxide
NOD	nucleotide oligomerisation domain
NPY	neuropeptide Y
PAMP	pathogen associated molecular pattern
PGRP	peptidoglycan recognition protein
PLA_2	phospholipase A_2
PML	polymorphonuclear leukocytes
rCRAMP	rat CRAMP
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
RTqPCR	real time quantitative PCR
SIC	streptococcal inhibitor of complement-mediated lysis
SLPI	secretory leukoprotease inhibitor
TGF	transforming growth factor
TLR	toll-like receptor
VDRE	vitamin D responsive element
wt	wild type

Introduction

A personal account

When I was in Medical School studying immunology and microbiology, the innate immune system was almost completely overlooked. Instead, we spent most of the time discussing adaptive immunity. The rearrangements of the genes encoding the T-cell receptor were studied in great detail. This was a complicated matter and I just barely passed the exam. I soon forgot about these molecular details, which I never completely understood. At that time, the idea of becoming a PhD-student seemed very far away. However, some years later, I came across a popular science article about antimicrobial peptides in insects, frogs and humans. The message was that these peptides were responsible for keeping us healthy and free from infections. I became immediately fascinated and couldn't get the topic out of my head. I got in touch with Birgitta and Gudmundur and became involved in a project on purification of antimicrobial compounds from human colon mucosa (Paper I). The robustness and simplicity of the extraction procedure were attractive and could be applied to virtually any biological material. I started to think of the brain and why this organ was so rarely infected. I read that the blood brain barrier (BBB) was responsible for the protection of the brain, but exactly how this was performed was not mentioned. Maybe there were antimicrobial compounds present in the brain as well? I made a peptide/protein extract of rat brain and surprisingly found potent antimicrobial activity. This finding was the starting point for the rest of my work. The continuation and how it all ended you can read about in this thesis.

Immunity

How can we stay healthy?

We are constantly exposed to overwhelming amounts of bacteria. They are present on all body surfaces, and still we are healthy most of the time. Obviously, there must exist potent mechanisms protecting us against this massive bacterial load. For a long time antibodies and specific T-cells of the adaptive immune system were in focus. The fact that insects and plants stay healthy, despite the lack of adaptive immunity was intriguing for the scientific community. This was the starting point for Hans G. Boman and coworkers in the early seventies when he investigated the immunity of the silk moth *Hyalophora cecropia.* The pupae of this insect provided a convenient model system due to their size. Large amounts of biological material could be isolated from this "test tube animal", making biochemical analyses possible. The pupae of Cecropia were injected with bacteria, hemolymph was isolated and after several years of hard work, the amino acid sequences of the first antimicrobial peptides (AMPs) could be reported (Steiner *et al.*, 1981). Later it was shown in Drosophila that a deletion of the gene encoding an antimicrobial peptide causes a massive fungal infection, demonstrating that Drosophila relies on a peptide-based defense system against infection (Lemaitre *et al.*, 1996). The pioneering work in insects was followed by work in macrophages and frogs showing that antimicrobial peptides constitute a vital part of innate immunity in species as diverse as insects, frogs and humans.

Immunity – an overview

Before entering the world of antimicrobial peptides, it is important to consider some general properties of immunity. Innate immunity comprises everything that is already in place, including epithelial cells, neutrophils and macrophages, as well as their effectors. The adaptive immunity, on the other hand, is based on B and T cells and their specific receptor repertoire. These two parts of the immune system are depending on each other to fulfill the ultimate goal: to maintain the integrity of the host. To illustrate the complementary roles of innate and adaptive immunity, an infectious process in a skin wound can be considered. Within minutes after a microbial assault, epithelial cells of the skin release antimicrobial and chemotactic compounds resulting in direct antimicrobial attack and in the recruitment of neutrophils to the site of infection. Dendritic cells in the sub-epithelial tissue sample material from the invading pathogen and migrate, via the lymphatic vessels, to a local lymph node. There it presents its "pray" via MHC molecules to T-cells with a specific T-cell receptor. Thus, by presenting the antigen, dendritic cells constitute an important

link between the innate and adaptive immunity. The activated T-cell clone multiplies – a process designated clonal expansion – and mediates a signal to B-cells, activating one clone that expands. This amplification procedure of adaptive immunity results in massive amounts of B- and T-cells specifically directed against the invading pathogen. The immediate effects of these events are that activated T-cells start to circulate and scan for the specific antigen to which they are primed. In addition, the activated B-cell clone differentiates into plasma cells, releasing large amounts of antibodies directed against the invader. The combined strategy of B- and T-cells results – in the majority of cases – in the elimination of the microbe. Moreover, some activated B- and T-cells become memory cells, thus providing life long immunity against the particular infectious organism (Janeway *et al.*, 2001).

Innate Immunity – the first line of defense

One weakness inherent to adaptive immunity is that it takes 3-5 days before T and B-cells are fully active. The growth rate of bacteria is very rapid, duplicating every 20 minutes, and the microbe would soon outnumber the host, if no action is taken. Fortunately, the innate immune system takes action and constitutes the first line of defense against invaders. It is always present and ready to operate. In order to fulfill this front line mission, the innate immune system is equipped with efficient sensors (receptors) with the capacity to recognize conserved structures of potential invaders or pathogens. When an invader is recognized, the host has access to potent bactericidal effectors, such as antimicrobial peptides.

How do we know that bacteria are present?

A sensing function for recognition of a pathogen is of vital importance. One type of sensors – or receptors – of the innate immune system are the toll like receptors (TLRs), which are present on the surface of epithelial cells, macrophages, dendritic cells and neutrophils. The TLRs recognize conserved molecules of microbes, collectively described as PAMPs (pathogen associated molecular patterns) (Janeway, 1989). TLRs were originally discovered in Drosophila (Hoffmann, 2003). The connection to mammalian innate immunity was established when the receptor for lipopolysaccharide (LPS) was shown to be identical to TLR-4 in mammals (Hoffmann, 2003). Today, 10 TLRs have been identified in humans. They recognize exclusively conserved microbial components, such as LPS, lipopeptides, unmethylated DNA (CpG) or double-stranded RNA, to mention just a few (Beutler *et al.*, 2003) (Fig 1). The advantage is that a limited number of receptors are able to recognize a large variety of molecular structures. In Drosophila, nine TLRs are

present, and eight of these are involved in development and only one in immunity (Hoffmann, 2003). This is in contrast to humans, where all TLR's have been implicated in immunity and none in development (Beutler, 2004).

Recently, additional sensors of microbial products have been identified, such as nucleotide oligomerisation domain (NOD) (Inohara *et al*., 2004) and peptidoglycan recognition protein (PGRP) (Steiner, 2004) (Fig 1). Interestingly, a mutation in the gene encoding the intracellular receptor NOD-2 has been associated with Crohn's disease (Kobayashi *et al.*, 2005). Moreover, receptors for bacterial formylated peptides (FPRs) have been shown to be important for defense against bacterial infection (Gao *et al.*, 1999). The scavenger receptor MARCO mediates defense against pneumococci and inhaled small particles (Arredouani *et al.*, 2004). Mannose Binding Lectin (MBL) and Lipopolysaccharide Binding Protein (LBP), represent circulating proteins with the capacity to recognize pathogens, by binding mannose and LPS, respectively (Weiss, 2003; Gadjeva *et al*., 2004).

The arsenal of innate immunity

Since the ultimate goal is to eradicate the invader, the host is equipped with efficient molecules, exhibiting direct microbial killing. In the circulation, a number of peptides and proteins have this capacity, such as lysozyme, Bactericidal permeability-increasing protein (BPI) and factors of the complement system. There are also "professional" phagocytes (macrophages and neutrophils), which engulf and destroy microorganisms. Ingested microbes end up in a specialized organelle, the phagosome, where they are killed by either oxygen-dependent or -independent mechanisms. The NADPH-oxidase requires oxygen and produce reactive oxygen species, such as superoxide, H_2O_2 and hydroxyl radicals, which efficiently kill microbes (Beutler, 2004). Antimicrobial peptides constitute the main oxygen-independent bactericidal mechanism of phagocytic cells and are stored in intracellular granules. These granules fuse with the phagosome, forming the phagolysosome. In this limited space, the antimicrobial peptides reach high concentrations (mg/ml) leading to rapid killing of engulfed microbes. Thus, the total arsenal of innate immunity poses a serious threat for invading microorganisms.

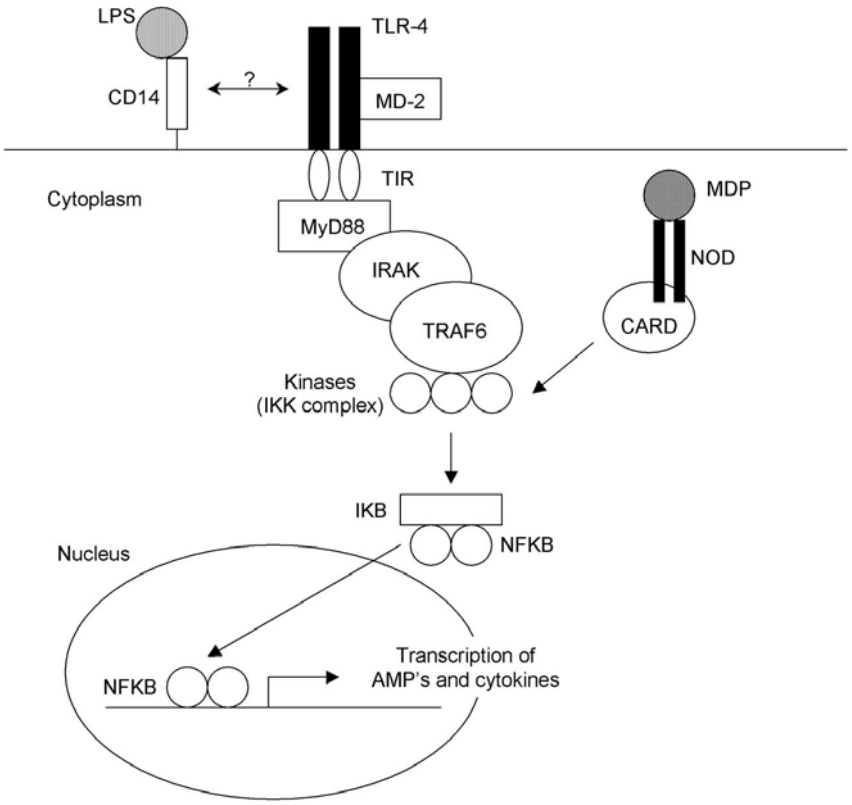

Figure 1. Toll like receptor signaling

TLR-4 receptor complex, including CD14, recognizes the bacterial product LPS. However, all events occurring in the initial binding are not known. Further downstream, the adaptor-protein MyD88 plays an important role of mediating LPS signaling, via IRAK and TRAF6 to the κB-complex. Degradation of IκB releases NFκB, which is translocated to the nucleus, where it binds to promoters of genes of innate immunity, including the antimicrobial peptides HBD-2 and HBD-3. The intracellular receptor NOD/CARD binds muramyl dipeptide (MDP) and activates genes via NFκB.

"Proof of concept" of Innate Immunity

The importance of innate immunity can be illustrated by human diseases or by gene deletions in mice (Qureshi *et al.*, 2003; Cook *et al.*, 2004). The loss of any arm of innate immunity leads to severe immunodeficiency. At the receptor level, this is demonstrated in mice with mutated genes encoding different TLRs, resulting in increased susceptibility to infection (Qureshi *et al.*, 2003). TLRs have also been implicated in non-infectious conditions, such as atherosclerosis (Tobias *et al.*, 2005), and autoimmunity (Rifkin *et al.*, 2005). The loss of cellular components in innate immunity, such as in neutropenia, may lead to sepsis caused by a number of bacteria, often involving commensal forms. The effector arm of innate immunity can also be affected by mutations, such as in chronic granulomatous disease, where the NADPH

oxidase is dysfunctional, leading to severe and life threatening infections (Palmblad *et al.*, 2005). The loss of antimicrobial peptides also causes increased susceptibility to infection (discussed later). Taken together, these findings illustrate that the host depends on the innate immune system in the defense against invading pathogens.

Innate immunity and immunological memory

The idea of vaccines is to create a long lasting immune reaction against a specific pathogen. Thus, immunity must be associated with memory. This is achieved by introducing small amounts of non-infectious antigen to the host; an event we call vaccination. However, the antigen alone is not sufficient to create an immunological memory. There is also a need for adjuvant. Charles Janeway has described this concoction as the immunologist's "dirty little secret", because of its unknown mechanism of action (Janeway, 1989). However, the secret of adjuvants has gradually become unraveled. A major breakthrough was achieved when it could be demonstrated that TLR-4 is needed for the adjuvant effect of LPS. The importance of TLRs for successful vaccination became apparent when non-responders to a Borrelia vaccine (Lyme disease) were shown to be deficient in TLR signaling (Alexopoulou *et al.*, 2002). Moreover, unmethylated DNA (CpG) has been suggested to contribute to the immunostimulatory effects of adjuvants via the stimulation of TLR-9 (Krieg, 2002). Thus, recent molecular findings regarding innate immunity explain old observations on the use of adjuvant in vaccine development (Germain, 2004).

Evolutionary aspects of innate immunity

The development of adaptive immunity appeared 550 million years ago, at early vertebrate times, and is shared by all vertebrates except jawless fish. What selective advantages have the adaptive immune system given to vertebrates? Why cannot vertebrates, like invertebrates, depend entirely on innate immunity? The reasons, according to Medzhitov and Janeway (1997), are simply that humans are different from insects. We live longer than insects and the immunological memory of adaptive immunity gives many advantages during a long life with the obvious risk of re-infection. Moreover, the amplification effect through clonal expansion of B- and T-cells is unique to adaptive immunity and beneficial for the host. However, since adaptive immunity evolved together with innate immunity, there are many links between the two systems. In fact, the adaptive immune system would be severely hampered without the activating and directional signals provided by cells of innate immunity (Medzhitov *et al.*, 1997). Taken together, innate and adaptive immunity have co-evolved and are functionally intertwined.

Antimicrobial peptides and proteins

Antimicrobial peptides are important effectors of innate immunity

The field of antimicrobial peptides (AMPs) originates from several different research disciplines. Early work focused on proteins of human neutrophils (Zeya *et al.*, 1966). The work on the bactericidal effects of neutrophils was fuelled by the discovery of Chronic Granulomatous Disease (CGD), a mutation of the oxidative burst, resulting in deficient bacterial killing. However, neutrophils of these patients still kill a substantial amount of bacteria *in vitro*, suggesting additional bactericidal mechanisms independent on oxygen (Lehrer, 2004). It was shown that several cationic proteins were responsible for this oxygen independent bacterial killing in neutrophils (Odeberg *et al.*, 1975). In 1981, Hans G. Boman and coworkers could report the first sequences of AMPs isolated from the hemolymph of *Hyalophora cecropia* (Steiner *et al.*, 1981). This was soon followed by the isolation of AMPs from rabbit macrophages (Selsted *et al*., 1983). Another milestone in this research field was when Michael Zasloff isolated and characterized magainins (Hebrew for "shield") in frog skin (Zasloff, 1987). Thus, around 1987, AMPs had been isolated from insects, frogs and mammals; and this was only the beginning. Since then, AMPs have been isolated from a number of different sources, ranging from plants, insects, fish and mammals, including humans. Today (01/11/2004) there are 880 AMPs identified (Tossi, 2005). This thesis will mainly deal with AMPs of human, rat and mouse.

General

In mammals there are two main families of AMPs, the defensins and the cathelicidins (Zasloff, 2002). The defensins form three structurally distinct groups: the α-defensins, which are found in neutrophils and Paneth cells of the small intestine, and the β-defensins, which are mainly synthesized by epithelial cells (Ganz, 2003). In addition, primates express circular theta defensins (Tang *et al.*, 1999). Cathelicidins consist of a conserved proregion, cathelin, and a variable C-terminal antimicrobial domain. Upon activation, the C-terminal domain is cleaved off, liberating the active antimicrobial peptide (Zanetti, 2004). LL-37, the single human cathelicidin is located in neutrophils (Gudmundsson *et al.*, 1996), expressed by several mononuclear cells (Agerberth *et al.*, 2000), and in various epithelia throughout the body (Bals et al., 1998; Frohm Nilsson et al., 1999). The cathelicidins in mouse and rat have also been characterized and named CRAMP (Gallo *et al.*, 1997) and rCRAMP (Termen *et al.*, 2003), respectively. The cathelicidin peptides in these three species are devoid of cysteine residues and folded in amphipathic α-helical structures (Zanetti, 2004).

Mechanism and structure

AMPs generally kill bacteria by destruction of their membranes, although there are examples of peptides with intracellular targets, such as PR39 and buforin (Brogden, 2005). There are two different models, which describe how AMPs destroy bacterial membranes. In the first model peptides integrate into the bacterial membrane, resulting in "holes" that can be described either as barrels (barrel-stave model) or toroidal pores. In the second model, peptides accumulate on the bacterial surface and form a "carpet", hence the name "carpet model". The peptides then integrate and dissolve the membrane in a detergent-like manner (Brogden, 2005).

There is no stereospecific interaction between AMPs and specific receptors, since AMPs composed of all-D amino acid residues are equally bactericidal as their naturally occurring all-L counterparts (Wade *et al.*, 1990).

Despite the differences in primary and secondary structure between defensins and cathelicidins, they share an amphipathic character (one hydrophobic side and one hydrophilic). This feature makes AMPs water soluble, while still maintaining the capacity to insert into lipid bilayers. Since most antimicrobial peptides are positively charged, they are prone to interact with the negatively charged outer leaflet of bacteria via electrostatic interaction. Both the degree of amphipathicity and the charge are important determinants for the antimicrobial activity (Tossi *et al.*, 2000).

AMPs preferentially interact with and lyse prokaryotic cells, although at high concentrations eukaryotic cells are also ruptured. The reason for this preference may be the presence of negatively charged molecules in prokaryotic membranes, such as phospolipids, lipotechoic acid and LPS. Eukaryotic membranes, on the other hand, are dominated by neutral zwitterions and cholesterol, resulting in a more neutral charge (Zasloff, 2002). Pathogenic bacteria have exploited this mechanism and are capable of reducing their surface charge by integrating cationic molecules into the membrane. Thus, some pathogens are less susceptible to the actions of AMPs and are more often implicated in disease (Peschel, 2002).

Biology of defensins

Alpha- and theta-defensins

Alpha-defensins (HNP 1-4) are found mainly in neutrophils, but also in mononuclear cells, such as NK-cells and γδ-T-cells (Agerberth *et al.*, 2000). These peptides are made as precursor proteins but are stored in their mature peptide forms in granules of neutrophils and other immune cells. The transcription and translation of α-defensins

occur mainly in the bone marrow, since no transcripts are detected in circulating neutrophils (Daher *et al*., 1988; Date *et al*., 1994). Interestingly, mouse neutrophils do not contain α-defensins (Eisenhauer *et al.*, 1992), whereas rat neutrophils are equipped with these peptides (Eisenhauer *et al.*, 1989).

The α-defensins, HD-5 and HD-6, are expressed in Paneth cells of the small intestine and in the genital tract. In the small intestine, HD-5 is processed by trypsin, while the processing in other tissues is unknown (Ghosh *et al.*, 2002). In the small intestine of the mouse, many α-defensins – designated "cryptdins" – are expressed. The processing of pro-cryptdins is mediated by matrilysin (see below) (Ouellette *et al.*, 2001).

Besides the antibacterial effects, α-defensins have been demonstrated to exert anti-HIV activity mainly by binding to surface structures of the virus and CD4 T-cells (Chang *et al.*, 2004). It has also been reported that α-defensins constitute the main component of CD8(+) antiviral factor (CAF), an endogenous factor released from CD8 T-cells with potent anti-HIV properties (Zhang *et al.*, 2002). This finding was later retracted, and the α-defensins detected in CD8 T-cells could be traced to neutrophils (Zhang *et al.*, 2004). Nevertheless, the fact that α-defensins are potent inhibitors of HIV infection remains undisputed (Mackewicz *et al.*, 2003).

The capacity to block HIV also applies to theta defensins, which function as lectins, binding to carbohydrate structures on viral and cell surfaces (Munk *et al.*, 2003; Wang *et al.*, 2003). The genes encoding these circular peptides are present both in macaque monkeys and humans. However, only monkeys express the peptide because of a mature stop codon in the human gene (Cole *et al.*, 2004). The lack of theta defensins in humans has been proposed to account for HIV susceptibility. The hypothesis that individuals resistant to HIV would express theta-defensin peptides as a result of a novel mutation was analyzed in sero-negative, highly exposed sex workers in Thailand. The outcome was that these individuals had no active transcription of theta defensins, suggesting the involvement of other endogenous protective mechanisms (Yang *et al.*, 2005).

Beta-defensins

The first β-defensin (HBD-1) was originally discovered in human blood filtrate (Bensch *et al.*, 1995), and later found to be expressed in epithelia (Zhao *et al*., 1996). This was followed by the discovery of β-defensin 2 (HBD-2) in psoriatic scales. The cellular source of HBD-2 was traced to keratinocytes of psoriatic patients (Harder *et al.*, 1997). HBD-1 and HBD-2 preferentially killed Gram-negative bacteria, while the crude psoriatic peptide/protein extract killed both Gram-negative and Gram-positive

bacteria. Hence, the search for a factor killing specifically Gram-positive bacteria was initiated. This strategy proved to be successful and HBD-3 was isolated (Harder *et al.*, 2001). At the same time, another German group could identify HBD-3 by means of computer-based cloning (Garcia *et al*., 2001a).

Subsequent cloning revealed that β-defensin genes encode a signal peptide followed by the mature peptide. Thus, there is no conventional proform of β-defensins, illustrating that epithelial cells are different from myeloid cells in this respect. Genomic analysis has located the β-defensin genes to a 1 Mb cluster on chromosome 8p22-23. This information was used to identify the fourth human β-defensin, HBD-4 and the highest expression levels were found in the testis (Garcia *et al*., 2001b), suggesting a role in reproduction. Recently, 28 human and 43 mouse β-defensin genes have been identified (Schutte *et al.*, 2002). Several of these newly described β-defensin genes are actively transcribed in the reproductory tract and brain (Maxwell *et al.*, 2003). Future work may reveal novel and unexpected functions for this large family of AMPs.

HBD-1 is constitutively expressed, while the other three "original" β-defensins are inducible by various inflammatory or infectious stimuli. The paradigm of antimicrobial peptide induction in humans is HBD-2, which is induced by cytokines, LPS and other bacterial products (Selsted *et al.*, 2005). The induction is dependent on TLR-2 and the transcription factor NFκB (Hertz *et al*., 2003; Vora *et al*., 2004). The cell surface receptors associated with expression of HBD-3 and HBD-4 remain to be determined. A recent study suggests that not only pathogens, but also T-cells induce the expression of β-defensins in epithelia. The T-cell derived cytokine IL-22 was recently found to specifically induce HBD-2 and HBD-3 in the skin, suggesting that the adaptive immune system plays a role in shaping innate defenses of epithelia (Wolk *et al.*, 2004).

HBD-1 and 2 have been shown to be chemotactic for immature dendritic cells and memory T-cells via the chemokine receptor CCR6 (Yang *et al.*, 1999). This was the first direct evidence that AMPs constitute an important link to the adaptive immune system. Notably, mouse β-defensin 2 (mBD-2) has been suggested to act as an adjuvant by stimulating cells via TLR-4 (Biragyn *et al.*, 2002). The implications of this finding would question the idea of TLRs as receptors exclusively for pathogenic products. Consequently, this finding has been disputed by the argument that trace amounts of LPS was bound to mBD2, and thus was responsible for the effect (Kopp *et al.*, 2002). However, the "danger model" argued for by Matzinger (2002), supports the notion that endogenous substances act as ligands for TLRs. This concept is illustrated by the fact that heat shock proteins, hyaluronan degradation products, oxidized LDL, surfactant protein A, saturated fatty acids and fibronectin have been shown to activate

TLR-4 (Lee *et al.*, 2003; Seong *et al.*, 2004). Recently, TLR-4 was shown to contribute to the development of neuropathic pain, independent of infection (Tanga et al., 2005). Thus, it appears that TLRs act as receptors both for "stranger" (exogenous) and "danger" (endogenous) molecules (Seong *et al.*, 2004).

"Proof of concept" for defensins

The first link between deficiency of AMPs and human disease was found in Cystic Fibrosis (CF) patients. The airway surface fluid (ASF) of CF patients contains high amounts of saline and was found to be poor in bacterial killing (Smith *et al.*, 1996). The high salinity of ASF from CF-patients was, in a later study, proposed to inactivate HBD-1 (Goldman *et al.*, 1997). Thus, the inactivation of one antimicrobial peptide was claimed to be responsible for the frequent lung-infections seen in CF patients. However, this issue is now described in terms of "the salt controversy", and the precise mechanism behind the innate immune deficiency in CF patients remains to be elucidated (Guggino, 1999; Donaldson *et al.*, 2003).

To obtain *in vivo* information on the role of defensins, KO-mice have been utilized. Mice deficient in β-defensin-1 expression did only exhibit a mild phenotype with regards to infectious susceptibility. Delayed clearance of *S. aureus* in the lung and urinary tract was observed in these mice compared to wild type (wt) controls (Morrison *et al.*, 2002; Moser *et al.*, 2002). These observations are interesting, since they illustrate the concept of redundancy among antimicrobial peptides.

The α-defensins in Paneth cells of mouse small intestine, the cryptdins, are processed to their mature forms by the metalloproteinase matrilysin, and mice deficient in this enzyme do not produce mature cryptdin peptides (Wilson *et al*., 1999; Ayabe *et al*., 2002). Interestingly, matrilysin-KO mice die more rapidly and at lower doses of orally administered *Salmonella typhimurium*. By preventing the processing of cryptdins, the non-redundant role of defensins in the antibacterial defense of the gut could be established (Wilson *et al.*, 1999). Apparently, mice die from *Salmonella typhimurium* infection, while this is not a lethal disease in humans. Could the difference be attributed to the presence of HD-5 in human Paneth cells? To address this question, a transgenic mouse expressing HD-5 in Paneth cells was generated. Surprisingly, this mouse was resistant to orally, but not systemically, administered *Salmonella typhimurium*, clearly demonstrating that HD-5 is a major protective factor against this pathogen in the gut (Salzman *et al*., 2003b). Interestingly, a link has been established between Paneth cell defensins and Crohn's disease (Wehkamp *et al.*, 2004). Mutations in the gene encoding the intracellular bacterial receptor NOD-2 were found among a subset of Crohn's patients (Hugot *et al.*, 2001). Later, it was demonstrated

that this mutation results in reduced defensin expression in Paneth cells (Kobayashi *et al.*, 2005). These findings suggest that deficient expression of defensins can lead to overgrowth of bacteria in the small intestine, and thus play a role in the pathogenesis of Crohn's disease.

Biology of cathelicidins

The other main family of AMPs in mammals is the cathelicidins. Cathelicidins have a conserved proregion, cathelin, in common, and a variable C-terminal antimicrobial domain. Upon activation, the C-terminal domain is cleaved off, liberating an active antimicrobial peptide (Zanetti, 2004). The conserved proregion exhibits 70 % sequence identity to cathelin, an inhibitor of the proteolytic enzyme Cathepsin L (cathelin is an acronym for cathepsin L inhibitor) (Zanetti, 2005). The term cathelicidins was proposed by Zanetti et al. in 1995 to describe precursor proteins that contain a cathelin-like sequence to which a cationic antimicrobial domain is connected (Zanetti *et al.*, 1995). This arrangement could be described as a garden tool with a common handle (cathelin part) and exchangeable tools (C-terminal antimicrobial domain) (Boman, 1996). Cathelicidins are represented in all mammals and exhibit a broad array of biochemical structures and genetic diversity between different species. In cows, pigs and sheep between 7-11 different cathelicidin genes are present, and the structure of the mature peptide can be α-helical, linear, proline-rich, or loop-like. This is in contrast to the situation in human, mouse and rat, having only one single cathelicidin gene, encoding an α-helical peptide, devoid of cysteine residues (Gudmundsson *et al.*, 1996). The variety of C-terminal antimicrobial domains is thought to reflect the different selection pressures under which each species has evolved (or, metaphorically, the different challenges in the garden). In this thesis, I will mainly focus on the human cathelicidin LL-37, and its orthologues, CRAMP in the mouse (Gallo *et al.*, 1997) and rCRAMP in the rat (Termen *et al.*, 2003).

Sites of expression and processing

The human cathelicidin LL-37 is located both in myeloid cells, and in various epithelia throughout the body (Gudmundsson *et al.*, 2004). The transcript of LL-37 was first detected in bone marrow and testis using northern blot analysis (Agerberth *et al.*, 1995). The transcript of LL-37 has since been detected in additional cell-types including monocytes, NK-cells, B-cells and γδ-T-cells (Agerberth *et al.*, 2000). In addition, mast cells (Di Nardo *et al.*, 2003), eosinophils and dendritic cells of the skin of newborns have been shown to contain the LL-37 peptide (Marchini *et al.*, 2002). A

number of epithelial tissues express LL-37, such as skin (Frohm *et al*., 1997; Dorschner *et al.,* 2001), colon (Hase *et al*., 2002; Schauber *et al.,* 2003), genital tract (Frohm Nilsson *et al.*, 1999) and the respiratory tract (Bals *et al.,* 1998; Agerberth *et al*., 1999). In addition, the mammary, salivary and sweat glands express LL-37 (Murakami *et al*., 2002a; 2002b and 2005).

The synthesis, storage and secretion of LL-37 appear to differ between myeloid cells and epithelial cells. In neutrophils, active transcription of LL-37 occurs mainly in the bone marrow, whereas circulating neutrophils contain the proform stored in granules. The processing of the proform occurs extracellularly and is mediated by proteinase-3 (Sorensen *et al.*, 2001).

In epithelial cells the events regarding transcription and storage are not fully elucidated. Keratinocytes have been shown to store LL-37 or the holoprotein in lamellar bodies (Braff et *al*., 2005a). The processing enzyme of LL-37 in epithelial cells remains unknown. In a recent report, several variants of LL-37 were found in sweat, indicating a complex cleavage pattern or involvement of promiscuous proteolytic enzymes (Murakami *et al*., 2004). In seminal plasma, the enzyme gastricsin processes the holoprotein to ALL-38 (Sorensen *et al.*, 2003b).

Since LL-37 is cytotoxic in high concentrations, there is a need for tight control of the release of this peptide (Johansson *et al.*, 1998). In plasma the concentration of the holoprotein hCAP/18 was measured by ELISA to be 1.18 µg/ml (Sorensen *et al.*, 1997). Interestingly, human plasma was found to completely block the antimicrobial activity of LL-37, and apolipoprotein A-I could be identified as the scavenger of LL-37 (Wang *et al*., 1998; Sorensen *et al*., 1999).

The gene encoding LL-37 is translated as a pre-proform, and after cleavage of the signal peptide the proform is processed to yield the cathelin part and the mature peptide, LL-37. The cathelin part was originally thought to act as a protease inhibitor, but recently it was shown that cathelin also exhibits antimicrobial activity. Thus, the processing of the proform results in two antimicrobial polypeptides: cathelin and LL-37 (Zaiou *et al.*, 2003).

The gene and its regulation

The gene encoding LL-37 consists of 4 exons, which is the same organisation as for all cathelicidins. The first three exons encode the signal peptide and the cathelin proform, while exon 4 encodes the processing site and the antimicrobial domain (Gudmundsson *et al.*, 1996) (Fig 2). The human gene for LL-37 is called *CAMP* (cathelin antimicrobial peptide) and is located on chromosome 3p21 (Gudmundsson *et*

al., 1995), which is homologous to mouse chromosome 9, where the gene encoding CRAMP is located (Gallo *et al*., 1997).

The promoter of the gene has several putative binding sites for known transcription factors, such as NF-IL6 and STAT-3, suggesting involvement of specific signal transduction pathways (Gudmundsson *et al*., 1996). Elevated expression of LL-37 was first detected in lesions of psoriatic patients (Frohm *et al.*, 1997). In addition, different bacteria induce the expression of the *CAMP* gene, which has been shown for *S. aureus* and GAS in keratinocytes (Dorschner *et al*., 2001; Midorikawa *et al*., 2003) as well as for *Helicobacter pylori* in gastric epithelial cells (Hase *et al.*, 2003). In contrast, *Shigella spp* down-regulates LL-37 in epithelial cells of the large intestine, an effect that can be mediated by bacterial DNA (Islam *et al.*, 2001).

Interestingly, the majority of cytokines do not affect LL-37 gene regulation. However, IGF-1, a growth factor involved in tissue regeneration of wounds, induced LL-37 expression in keratinocytes (Sorensen *et al.*, 2003a).

Cell differentiation has been proposed to be the main determinant of LL-37 expression, as demonstrated in colonic epithelial cells (Hase *et al.*, 2002). However, in another study, the induction of LL-37 by butyrate could be uncoupled from differentiation, indicating a specific effect of butyrate on the *CAMP* gene (Schauber *et al.*, 2003).

In the promoter of the LL-37 gene, putative binding sites of several transcription factors have been identified (Gudmundsson *et al.*, 1996). However, limited evidence exists for the involvement of any of these transcription factors in the regulation of LL-37 *in vivo*. In the rare congenital disorder Specific Granule Deficiency (SGD) the patients are extremely susceptible to bacterial infections. One explanation may be the lack of LL-37 and other AMPs (Ganz *et al*., 1988; Gombart *et al*., 2001). Some of these patients exhibited a mutation in the gene for the transcription factor c/EBP, suggesting a link to the expression of LL-37 (Gombart *et al*., 2001).

A genomic approach has identified Vitamin D-responsive elements (VDRE) in the promoter of the *CAMP* gene. This VDRE is functional *in vitro,* since Vitamin D induces the gene in keratinocytes (Wang *et al*., 2004; Weber *et al.,* 2005). Recently, it was shown that Vitamin D induces expression of *CAMP* mRNA in acute myeloid leukemia, keratinocytes, colon cancer cell lines and in human bone marrow derived macrophages (Gombart *et al*., 2005).

Interestingly, the involvement of reactive oxygen species (ROS) have been proposed to regulate expression of the mouse cathelicidin CRAMP in macrophages (Rosenberger *et al.*, 2004). In addition, hypoxia-inducible factor 1, α subunit (HIF-1α) was recently shown to regulate mouse CRAMP expression in myeloid cells

(Peyssonnaux *et al*., 2005). If these findings hold true, the strict division between oxygen-dependent and oxygen-independent bacterial killing needs to be reconsidered.

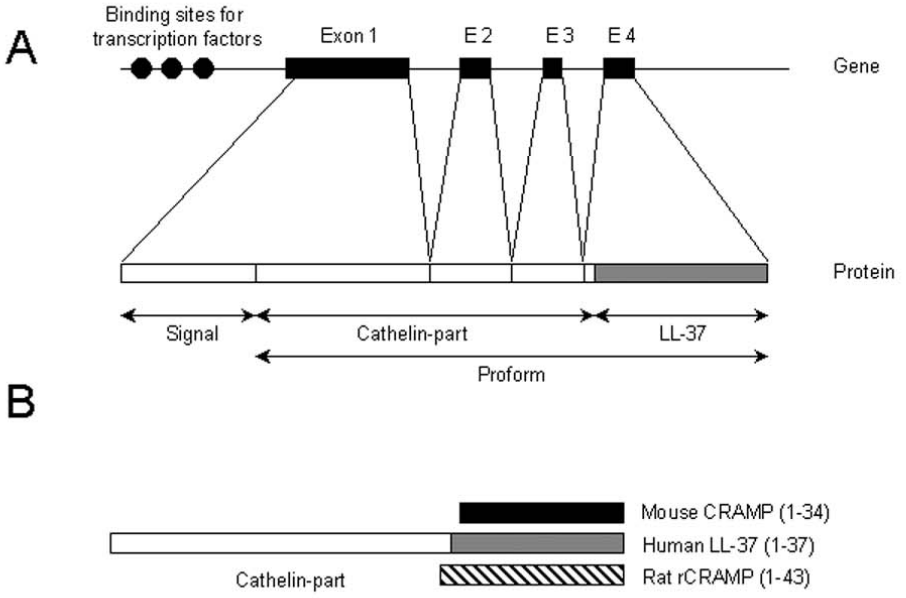

Fig 2. (A) The gene encoding LL-37 (B) Cathelicidin peptides in mouse, man and rat

(A) *The gene encoding LL-37 consists of 4 exons, where the first three encode the signal peptide and cathelin, whereas the fourth exon encodes the mature peptide LL-37.* ***(B)*** *The precursor including the cathelin part is processed by tissue- and species specific proteolytic enzymes. The mature forms in mouse (34 aa), human (37 aa + additional forms) and rat (43 aa) have different processing sites.*

Bacterial spectrum and mechanisms of resistance

LL-37 has been shown to exhibit bactericidal activity against a wide range of Gram-negative bacteria, e.g. *P. aeruginosa, S. typhimurium and E. coli,* as well as Gram-positive bacteria, such as *S. aureus, S. epidermidis and L. monocytogenes* (Turner *et al*., 1998). In addition, LL-37 kills fungi (Dorschner *et al*., 2004), virus (Howell *et al*., 2004) and parasites (Johansson *et al.*, 1998), whereas *Chlamydia* spp are intriguingly resistant to LL-37 (Donati *et al*., 2005; Edfeldt *et al*., 2005). Interestingly, and in contrast to the situation of many defensin peptides, LL-37 is active at high salt concentrations (Johansson *et al.*, 1998). Thus, LL-37 constitutes a complement to the defensin peptides at mucosal surfaces with variable salt concentration.

In physiological salt solution, LL-37 forms a stable α-helical structure. The extent of helicity correlates with the antibacterial activity (Johansson *et al.*, 1998). Two

different models have been proposed to explain the mechanism of action. Upon contact with bacteria, the LL-37 peptide accumulates on the membrane according to the carpet-model (Oren *et al.*, 1999), or forms toroidal pores in the lipid bilayer according to the model proposed by Henzler Wildman and coworkers (2003). Both mechanisms result in disruption of the membrane and lysis of bacterial cells. However, the exact mechanism *in vivo* remains unknown, possibly reflecting a very rapid process and the technical difficulties inherent to these biophysical studies.

The development of bacterial resistance against AMPs was first thought to be a rare event. However, there is now solid evidence that such resistance is widely spread among bacteria and is correlated to virulence. There are several ways in which bacteria can avoid the action of AMPs, including cell surface alterations, pump mechanisms and external trapping (Nizet, 2005). In contrast to many other bacterial strains, the Gram-positive bacterium *S. aureus* is quite resistant to AMPs. This is in part due to the frequent incorporation of D-alanine in the teichoic acid of the cell wall. The D-alanylation exposes positively charged amino groups, which in turn make the surface charge of the bacteria less negative (Nizet, 2005). Thus, the initial electrostatic attraction of the AMP to the bacterial cell surface is perturbed. A number of other Gram-positive bacteria such as Group B streptococci (GBS) and *Listeria monocytogenes* use this mechanism to avoid the action of AMPs (Peschel, 2002). Bacteria also secrete proteins that bind and neutralize AMPs, which has been demonstrated for GAS. This bacterium secretes SIC that has the capacity to bind LL-37 and human α-defensin (Frick *et al.*, 2003). *Neisseria gonhorreae* is equipped with efflux pumps that actively remove AMPs, such as LL-37 and protegrin, from the cytoplasm of the bacterium (Shafer *et al.*, 1998).

Additional functions

In addition to the antimicrobial effects, LL-37 exhibits a number of other activities. LL-37 has prominent chemotactic activities on polymorphonuclear leukocytes (PMLs), monocytes and CD4 T-cells (Agerberth *et al*., 2000; De Yang *et al.*, 2000). This activity was later shown to be mediated via Ca^{2+}-signaling and the formyl peptide receptor like-1 (FPRL-1) (De Yang *et al.*, 2000). Recently, the same group reported that mouse CRAMP (1-39) was chemotactic for PMLs and this effect was also mediated via FPRL-1 (Kurosaka *et al.*, 2005). Serum abrogates the antimicrobial activity of LL-37, but not ligation to the receptor FPRL-1, suggesting that specific activities of LL-37 can be linked to different parts of the peptide (De Yang *et al.*, 2000). LL-37 is also chemotactic for mast cells independently of FPRL-1, indicating the existence of additional receptor(s) (Niyonsaba *et al.*, 2002). In fact, LL-37 has

been shown to mediate release of IL-1β from monocytes via an additional receptor, P2X(7) (Elssner *et al.*, 2004).

Further, a number of studies have been carried out, stimulating immune cells with LL-37. Several genes in mouse macrophages and epithelial cells were affected by LL-37 stimulation (Scott *et al.*, 2002). An extension of this study demonstrated that LL-37 exhibited profound effects on the maturation of dendritic cells (Davidson *et al*., 2004), an effect independent of FPRL-1. This suggests a role for LL-37 as a potent immunomodulatory molecule, acting as a link between the innate and adaptive immune systems. LL-37 was recently found to up-regulate CD86 and HLA-DR in monocyte-derived dendritic cells (MDDCs) (Bandholtz *et al.*, 2005). In another study, MDDCs were shown to express LL-37, and the secretion from these cells was increased in atopic eczema after stimulation with the fungus *Malasezzia sympodialis* (Agerberth *et al.*, 2005).

The expression of LL-37 is induced in inflamed skin (Frohm *et al.*, 1997), but also after sterile incision, suggesting an involvement in the healing process (Dorschner *et al.*, 2001). In mice deficient in the gene encoding the cathelicidin CRAMP, GAS was found to induce larger wounds than in wt control mice (Nizet *et al.*, 2001). Later, the induction of LL-37 in keratinocytes was shown to be mediated via IGF-1 and TGF-α, growth factors involved in the wound healing process (Sorensen *et al.*, 2003a). Chronic ulcers lack LL-37, which also indicates that LL-37 is important in the healing process. Indeed, the blocking of LL-37 with specific antibodies delayed healing in a wound closure model (Heilborn *et al.*, 2003). This study provides direct evidence that LL-37 exerts wound healing functions. The formation of new blood vessels is vital for wound healing as well as for the development of solid tumors. LL-37 induces angiogenesis by a direct effect on endothelial cells mediated by FPRL-1 (Koczulla *et al.*, 2003). This finding could be confirmed *in vivo* both in a rabbit model of angiogenesis and by utilization of KO-mice.

“Proof of concept” for cathelicidins

Several of the initial studies in the field of AMPs were restricted to *in vitro* systems. The question was whether these peptides did play a role *in vivo*. During recent years, the role of AMPs as key effectors of innate immunity has been firmly established. Solid evidence has originated from human disease as well as from animal models. In humans, a potent up-regulation of LL-37 was observed in psoriasis (Frohm *et al.*, 1997), and the lesions of these patients are rarely infected, while patients with atopic dermatitis (AD) often suffer from bacterial infections in their eczemas. Recently, it was confirmed that psoriatic lesions contain higher levels of LL-37 and HBD-2, than

eczemas of AD (Ong *et al.*, 2002). Thus, the deficient AMP expression in AD may, in part, explain the high infection rate seen among these patients.

In a rare neutropenic disease, morbus Kostmann, patients have a low number of neutrophils and suffer from lethal infections. Treatment with recombinant granulocyte-colony stimulating factor increases the number of neutrophils to normal levels. However, even after this treatment, neutrophils have reduced levels of LL-37 and alpha-defensins in neutrophils. Moreover, saliva of Kostmann patients did not contain LL-37, which may explain why these patients frequently suffer from periodontal disease (Putsep *et al.*, 2002).

The development of mice deficient in the CRAMP-gene made it possible to test whether cathelicidins play a role *in vivo*. The first report using CRAMP-KO mice demonstrated that these mice suffer from larger wounds after GAS infection (Nizet *et al.*, 2001). In the same study, a CRAMP-resistant GAS mutant, induced larger wounds than the wt GAS, demonstrating that CRAMP resistance is connected to virulence *in vivo*. CRAMP-KO mice have been used to demonstrate non-redundant functions of CRAMP in *Salmonella typhimurium*-infected macrophages (Rosenberger *et al.*, 2004), in vaccinia infection (Howell *et al.*, 2004) and in studies of mastcell function (Di Nardo *et al.*, 2003). Recently, CRAMP-KO mice were used to show the role of CRAMP in the protection against invasive *Citrobacter* infection in the colon (Iimura *et al.*, 2005). Furthermore, CRAMP-KO mice were used to confirm data on the role of LL-37 in angiogenesis (Koczulla *et al.*, 2003).

Alpha-defensins	*aa*	*pI*	*Mol. weight*
HNP-1	30	8.68	3442
HNP-2	29	8.67	3371
HNP-3	30	8.33	3386
HNP-4	34	8.43	3324
HD-5	31	8.95	3311
HD-6	30	8.29	3390
Beta-defensins	*aa*	*pI*	*Mol. weight*
HBD-1	36	8.87	3928
HBD-2	41	9.30	4328
HBD-3	45	10.08	5155
HBD-4	49	9.45	5853
Cathelicidins	*aa*	*pI*	*Mol. weight*
LL-37	37	10.61	4493
rCRAMP	43	10.51	5031
CRAMP (1-34)	34	10.22	3878
CRAMP (1-38)	38	10.46	4306

Table 1. Biochemical properties of human defensins and cathelicidins of man, rat and mouse.

Antimicrobial proteins

During recent years there have been overwhelming amounts of data on "classical AMPs", i.e. defensins and cathelicidins, but also antimicrobial *proteins* have to be considered in this context. Already in 1922, Alexander Fleming isolated the first antimicrobial protein, lysozyme, from human saliva (Fleming, 1922). Recently, it was shown that Psoriasin, an 11 kD protein, is the main *E. coli* killing factor in human skin (Glaser *et al.*, 2005). The mechanism of action was suggested to be the capacity of Psoriasin to bind zinc, and thus making this essential nutrient unavailable for bacteria. The zinc sequestering mechanism is shared with Calprotectin, an antimicrobial protein that constitutes 60% of the granule content in human neutrophils (Berntzen *et al.*, 1990). Many body fluids contain antimicrobial proteins, such as phospholipase A_2 (PLA_2), the main bactericide in human tears (Qu *et al.*, 1998) and lactoferrin, a major bactericidal factor in human breast milk (Morrow *et al.*, 2004). Furthermore, Secretory leukoprotease inhibitor (SLPI) has been isolated from breast milk and cerebrospinal fluid (Doumas *et al.*, 2005). Myeloid cells also contain numerous antimicrobial proteins such as Eosinophilic cationic protein (ECP) (Venge *et al*., 1999), SLPI (Tomee *et al*., 1998) and BPI (Elsbach *et al.*, 1998). NK-cells

express the cytotoxic and bactericidal 9 kD protein granulysin, which has been proposed to be important in the defense against *Mycobacterium tuberculosis* (Stenger *et al.*, 1998).

In the search for AMPs in tissue extracts, histones and ribosomal proteins are frequently detected as antimicrobial compounds in the antibacterial assay. These proteins are highly cationic and exhibit bactericidal activity *in vitro*. Antimicrobial activity for histones was reported already in 1966 (Zeya *et al.*, 1966). Nevertheless, the close connection to chromatin structure and DNA packaging made them overlooked as true antimicrobial compounds for a long time. However, recent data suggest that histones are released from cells undergoing apoptosis (Rose *et al*., 1998). In addition, they can be processed into more active forms, as shown for buforin (Kim *et al.*, 2000).

A novel, 47-residue, antimicrobial peptide was isolated from human sweat and designated dermcidin (Schittek *et al.*, 2001). This peptide has no homology to peptides of the defensin or cathelicidin families and is active against bacteria in a wide range of salt conditions. Interestingly, the dermcidin gene was found to be up-regulated in invasive tumors and also to be expressed in the brain, suggesting novel and unexpected functions of this peptide (Porter *et al.*, 2003). It is noteworthy that also hepcidin, a key regulator of iron metabolism, was isolated from urine and identified on the basis of its antimicrobial properties (Ganz, 2004). Thus, antimicrobial activity may not be the main function of a peptide or protein, just a pecularity of nature to tell us that specific activities may not always be as well understood as we tend to believe.

Considering the stories of dermcidin and hepcidin, it is evident that the definition of AMPs or proteins constitutes a complex task. An emerging picture is that peptides or proteins with established functions turn out to exhibit potent bactericidal activity. I will here mention three examples of this emerging concept: chemokines, complement factors and neuropeptides.

Antimicrobial peptides and chemokines exhibit many similarities regarding structure, charge and size. Consequently, 11 chemokines were investigated for their antimicrobial activity and three of them were potent killers of bacteria in a defensin-like manner (Cole *et al*., 2001). The prominent feature of the active chemokines was that the C-terminal part was positively charged. The activity could be abrogated in high-salt buffer, similar to the α-defensins. Moreover, 17 out of 30 additional chemokines were analyzed and found to exert antimicrobial activity *in vitro* (Yang *et al.*, 2003).

The complement system consists of circulating peptides and proteins with many functions. One is to bind microorganisms, thus labeling them for phagocytosis, opsonisation (Janeway *et al*., 2001). It was recently shown that proteins of the complement system, anaphylatoxin 3a and its derivative C3-desArg, have the capacity to kill both Gram-negative and Gram-positive bacteria. (Nordahl *et al*., 2004).

The nervous system has direct influence on the function of innate immunity via hormones and their receptors, which are present on immune cells (Steinman, 2004). In addition, emerging evidence suggests that neuropeptides, which are important messenger molecules in the central and peripheral nervous system, exhibit direct antimicrobial effects. In a recent review article, substance P was proposed to contribute to innate immunity in the skin and in the mouth (Brogden *et al.,* 2005). Furthermore, neuropeptide Y (NPY) has been shown to be a potent killer of various bacterial strains (Vouldoukis *et al*., 1996; Shimizu *et al*., 1998). Interestingly, NPY is expressed in so called ensheathing cells surrounding the olfactory nerve on its way through the cribriform plate, separating the nasal cavity from the brain (Ubink *et al.*, 2000). Since the brain is rarely infected via the olfactory route, despite massive amounts of inhaled bacteria, there must exist efficient protective mechanisms. Recently, NPY was suggested to play a role in the protection of the brain and olfactory nerve against invading pathogens (Brogden *et al.,* 2005).

Taken together, these novel findings of additional functions for cytokines, complement factors and neuropeptides have substantially increased the number of antimicrobial peptides or proteins. However, there is a lack of direct evidence for their true involvement in innate immunity *in vivo.* Future studies involving loss-of-function models, i.e. KO-mice, will give further insight into these issues.

Pathogenic Neisseria

To put cathelicidin AMPs in a functional context, I have worked with *Neisseria meningitidis* (meningococci) and *Neisseria gonorrhoea* (gonococci). These bacteria belong to the same bacterial taxon line, but cause different types of disease. They share about 80% of their genomes. Meningococci colonize the nasopharynx of 10-15 % of healthy individuals. For reasons that remain unknown, meningococci sometimes invade the host, multiply in the blood and cause sepsis. In cases with severe bacteraemia, crossing of the BBB may occur, which leads to meningitis. The onset of disease is rapid, and a fatal outcome occurs in 15-20% of infected patients. Vaccination has only been partly successful because of the antigenic variation of this bacterium. There is vaccine available against the serotypes A and C, but not against serotype B, which were responsible for the recent outbreaks. Endemic outbreaks of meningococci still constitute a major problem among pilgrims and in military barracks (Rosenstein *et al.*, 2001). A serious meningitis epidemic hit Burkina Faso in 1996 that resulted in 4.363 fatal cases out of 42.907 affected individuals, clearly demonstrating that this pathogen still poses a major health problem (http://medilinkz.org/HealthTopics/diseases/meningitis/meningitis.asp). *Neisseria gonorrhoeae* causes the sexually transmitted disease gonorrhea, which may be responsible for sequelae in the form of scarring of oviducts resulting in infertility. There was a steady decline in the incidence of gonococcal infections, but during recent years there has been an increase in the number of infected individuals. The pathogenesis of these two infections is similar with three defined steps: adherence, attachment and invasion. Several important virulence factors have been characterized.

Adherence

Both *N. gonorrhoeae* and *N. meningitidis* express protruding structures named pili, consisting of multiple subunits of PilE-proteins. Pili are the "antennas" of *Neisseria* and are used to attach to host cells. Both meningococci and gonococci vary their pili by recombinatory events in the gene encoding PilE (Meyer *et al.*, 1984; Serkin *et al.*, 1998). This phase variation - or "changing of coats" - is considered to be of importance to avoid recognition by specific antibodies. Consequently, *Neisseria* infection does not lead to immunity (Hobbs *et al.*, 1999). Pili are important in *Neisseria* pathogenesis, since clinical isolates of both meningococci and gonococci are always piliated (Pujol *et al.*, 2000). The major adhesive protein of pili, PilC, is located at the top and at the basis of pili. A mutation of this protein results in piliated, but non-adhesive strains (Nassif *et al.,* 1994; Rudel *et al.,* 1995; Rahman *et al*., 1997).

Attachment and Invasion

The second phase of *Neisseria* pathogenesis involves a close interaction of the bacterium with host cells. This interaction is mediated via Opa-proteins and specific host receptors, such as sulphate proteoglycans (van Putten *et al.*, 1995) or CEACAM-receptors on the surface of epithelial cells (Virji *et al.*, 1996). The receptor interaction leads to internalization of bacteria into epithelial cells. *Neisseria* may avoid phagolysosomal killing and escape the cell via the basolateral side, thus causing disseminated disease (Mosleh *et al.*, 1997).

CD46

The primary function of the cell surface protein CD46 is to prevent complement activation on host cells. It has been shown that several pathogens, such as adenovirus, human herpes virus 6, measles virus and streptococcus pyogenes (GAS) utilize CD46 as a receptor (Cattaneo, 2004). In addition, CD46 has been proposed to be a receptor for pathogenic Neisseria, interacting with pilE and pilC (Kallstrom *et al.*, 1997). However, recent studies suggest that the role of CD46 is more complicated than previously anticipated. Recently, it was shown that despite the knock-down of CD46 with RNA interference, piliated strains of gonococci bind to the cell surface, suggesting involvement of additional receptor(s) (Kirchner *et al.*, 2005a and 2005b).

Other virulence factors

Porins are common outer membrane proteins of *Neisseria* and have been shown to induce immune responses via TLR-2. In addition, porins interfere with the complement cascade as an immune escape mechanism (Massari *et al.*, 2002).

In meningococcal disease, LPS is responsible for the damage of epithelial and endothelial cells (Dunn *et al.*, 1995). LPS of meningococci is slightly different from that of other Gram-negative bacteria, and is therefore referred to as lipooligo-saccharide (LOS). Strains deficient in LOS have impaired adherence and induce lower levels of cytokines in animal models (van der Ley *et al.*, 2003).

Iron is an essential nutrient for pathogenic bacteria. In humans the supply of iron is strictly regulated and in infectious conditions, available iron levels are decreased. However, meningococci have evolved several iron acquisition systems, which enable them to use human transferrin, ferritin and haemoglobin as iron sources (Larson *et al.*, 2004). The iron sources of mice and rats are not equally well accepted by meningococci, which may be one explanation why Neisseria strictly infect and cause disease in humans.

Animal models for meningococcal infection

To cause disease in mice or rats, pretreatment with iron dextran prior to meningococcal inoculation has been investigated. This proved to be a successful, although artificial way of studying meningococcal disease in animals (Yi *et al.*, 2003). A more physiological approach involves infant mice or rats, which because of their immature immune system are susceptible to meningococcal infection. However, vaccine studies require a mature immune system with the capacity to induce immunological memory. Another drawback of using infant mice or rats is that infection could not be demonstrated after 10 days of age (Mackinnon *et al.*, 1992). An animal model that better represents human neisserial infection could facilitate the development of novel vaccines against meningococcal disease.

Antimicrobial defenses of mucosal surfaces and brain

To put AMPs in a physiological context, I will briefly discuss their role at three different anatomical locations in the body: the colon, the cervix and the brain. The mucosal surfaces of colon and cervix have a normal flora, which is beneficial to these tissues. Considering the amount of bacteria present in the colon and also in the cervical area, the need for potent innate defenses is obvious. Indeed, epithelial cells of both colon and cervix are rich sources of antimicrobial peptides and proteins (Quayle, 2002). The brain, on the other hand, is not normally exposed to bacteria. However, recent data have demonstrated that the brain is capable of responding to microbial assault by activation of its own innate immune system, consisting of both neurons and microglial cells in a coordinated network (Polazzi *et al.*, 2002).

Human colon

Epithelial cells of the colon are protected by a thick layer of mucus, i.e. the glycocalyx (Lamont, 1992), and these cells constitute both a physical and chemical barrier to invading microorganisms (Fig 3). Colonic epithelial cells secrete a number of antimicrobial components, such as lysozyme, lactoferrin, SLPI, peroxidase and secretory PLA_2 (Lamont, 1992). Furthermore, epithelial cells constitutively express the human β defensin HBD-1, while HBD-2 is induced by LPS and other proinflammatory molecules (O'Neil *et al.*, 1999). The human cathelicidin LL-37 is constitutively expressed in differentiated cells near the top of the crypts of Lieberkühn (Islam *et al*., 2001; Hase *et al*., 2002; Schauber *et al.,* 2003). The fatty acid butyrate, a fermentation product of dietary fibers, induces the expression of LL-37 in colonic epithelial cell-lines (Hase *et al*., 2002; Schauber *et al*., 2003). Thus, the colon is well protected against invasive microbes by potent bactericidal effectors. However, a number of microbes have developed strategies to counteract these defenses. *Shigella spp* have been shown to down-regulate LL-37 and HBD-1 in colonic epithelial cells (Islam *et al.*, 2001). Similarly, *Salmonella typhimurium* causes down-regulation of an α-defensin in Paneth cells of the small intestine of mice (Salzman *et al*., 2003a).

Since both bacterial commensals and pathogens express conserved structural patterns, such as LPS and peptidoglycans, it is difficult to understand how the immune system can distinguish between friend and foe. If the commensals would activate the immune system in the same manner as pathogens do, inflammation would be the rule. Fortunately, inflammation is not prevailing and, in fact, recent data support a state of hyporesponsiveness in the colon (Melmed *et al.*, 2003). For instance, colon epithelial cells do not respond to LPS, despite the expression of TLR-4 (Hornef *et al.,* 2002). One explanation could be the recent finding that TLR-4 in colon is an *intra*cellular

receptor expressed in the Golgi apparatus (Hornef *et al*., 2002 and 2003). The shielding of pattern recognition receptors may be one way for the cell to avoid activation by non-invasive bacteria, such as commensals (Backhed *et al.*, 2003). Pathogenic bacteria may also actively modulate signal transduction pathways. This has been shown for nonvirulent *Salmonella* that blocks the degradation of the NFκB inhibitor IκB-α and thus suppresses proinflammatory signaling (Neish *et al.*, 2000). HBD-2 is induced in oral epithelial cells by a commensal strain via an NFκB independent mechanism, while pathogenic bacteria activated NFκB and thus the proinflammatory program in the host cell (Chung *et al.*, 2004). Furthermore, in the small intestine, angiogenin-4 is induced by *Bacteroides thetaiotaomicron*, a member of the gut microflora (Hooper *et al.*, 2003). Thus, commensal bacteria may induce specific antimicrobial peptides, without eliciting inflammation, suggesting that intestinal commensal bacteria influence gut microbial ecology and form local innate immunity.

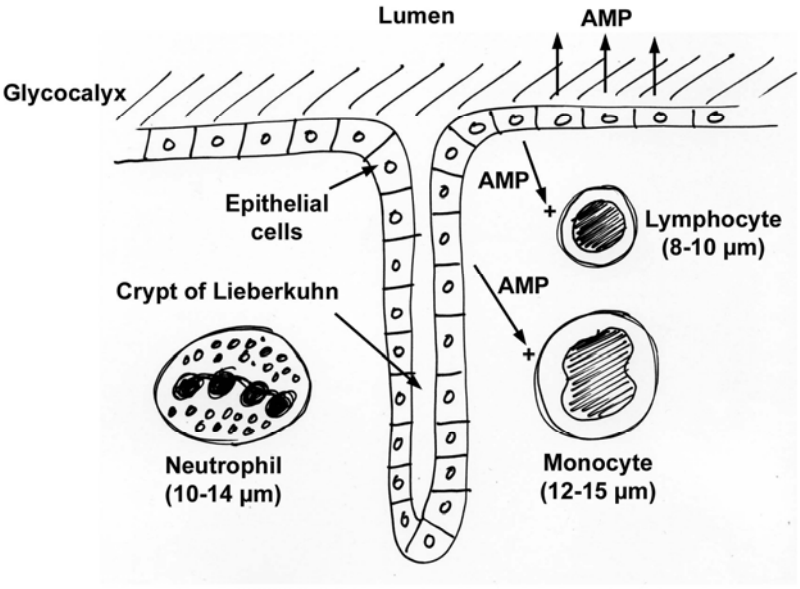

Figure 3. Innate imunity of the colon.

The colon epithelium is protected by a thick mucus-layer (glycocalyx). Antimicrobial peptides (AMPs) are produced by colon epithelial cells and secreted into the lumen of colon, where they kill bacteria. In addition, basolateral secretion of AMPs recruits immunecells to the site of infection.

Female genital tract

The female genital tract shares many immunological features with the intestine. The lower part of the female genital tract contains a normal flora, while the upper part, the cervix and uterus, normally are sterile (Fig 4). A special function of the female genital tract is to permit sperm, while microbial invaders should be eradicated.

The vaginal microflora constitutes a vital part of the first line defense of the lower genital tract. The dominating bacterial species are lactobacilli, which metabolize glycogen released from the vaginal epithelial cells into lactic acid, thus lowering the pH to 3.5-5. A disturbed normal flora, which may occur after antibiotic treatment, is a risk factor for subsequent infections (Quayle, 2002). Vaginal epithelial cells are weak responders to pathogens, suggesting that this tissue – like colon – is hyporesponsive (Fichorova *et al.*, 1999).

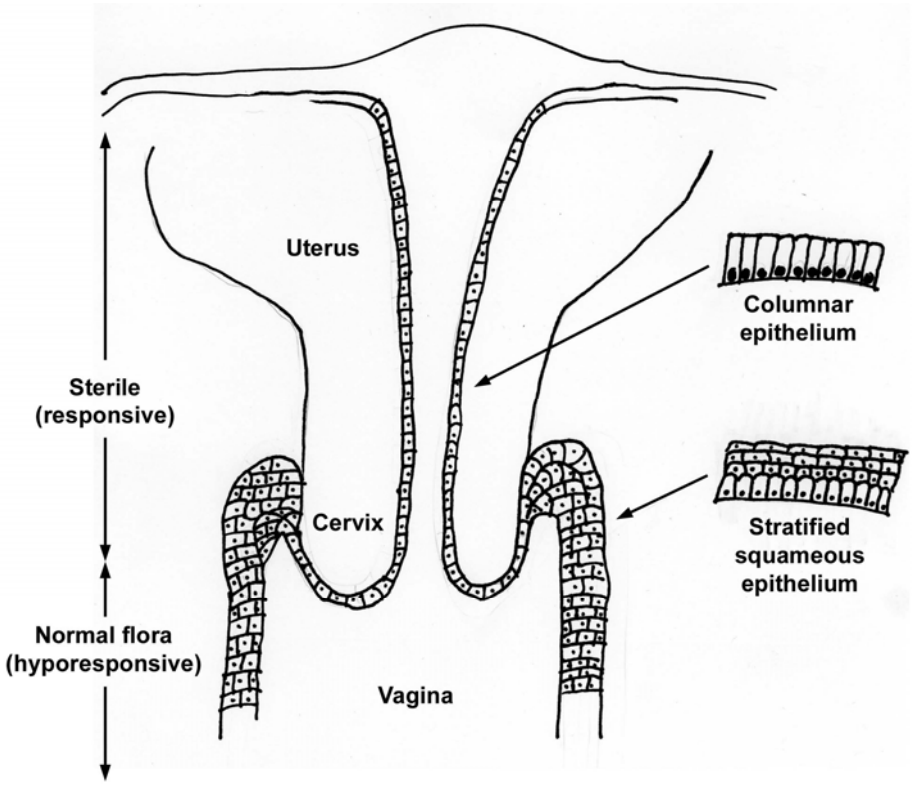

Fig 4. Female genital tract

The female genital tract can be divided in the non-sterile lower part (vagina) and the sterile upper part (uterus). The epithelial cells of the vagina are hyporesponsive to inflammatory stimuli, while the epithelia of uterus and cervix have the capacity to synthesize and release several AMPs, such as HBD-1, HD-5 and LL-37.

This is in contrast to cervical epithelial cells, which respond vigorously to invading pathogens by secreting mucus with potent antimicrobial activity. The mucus contains secretory IgGs, enzymes and antimicrobial peptides/proteins, including lysozyme and lactoferrin. In addition, cervical cells express the defensins HBD-1 (Valore *et al.*, 1998) and HD-5 (Quayle *et al.*, 1998) as well as the human cathelicidin LL-37 (Frohm Nilsson *et al.*, 1999). The antimicrobial activity of the cervical mucus varies during the menstrual cycle, with the most potent activity around ovulation (day 14) (Eggert-Kruse *et al.*, 2000). Thus, the aseptic state of the upper female genital tract, i.e. cervix and uterus, is maintained by a powerful innate immune system.

However, there are microbes, which have evolved mechanisms to avoid the host response. The human pathogens *Chlamydia trachomatis* and *Neisseria gonorrhoea* are two examples. Chlamydia infects cervical epithelial cells and is recognized via TLR-2, but not by TLR-4 (Darville *et al.*, 2003). Also Gonococci affect host cells independently of TLR-4 (Fichorova *et al.*, 2002). Some gonococcal strains contain efflux systems, which actively secrete AMPs out of the bacterial cytoplasm (Shafer *et al.*, 1998). Gonococcal strains with this efflux-mechanism exhibited increased virulence in a mouse model of goncococcal infection (Jerse *et al.*, 2003). This suggests that bacterial resistance to cationic AMPs, such as LL-37, is connected to bacterial virulence *in vivo*.

The brain

The brain is rarely infected and this has been ascribed to the protective properties of the BBB, shielding the brain parenchyma from the circulation. The BBB consists of endothelial cells connected with tight junctions. Beneath this layer there are astrocytes with extended foot-processes (podocytes), covering the basal part of the endothelial layer and providing a second cell layer (Goldstein *et al.*, 1986) (Fig. 5). In addition, to the BBB, there exists an intrinsic and well-developed immune system in the CNS (Carson *et al.*, 1999; Becher *et al.*, 2000). Important cells in CNS immunity are microglia (Vilhardt, 2005), but also astrocytes (astroglia) and oligodendroglia have been implicated in CNS immune reactions (Bergmann *et al*., 2004; Speth *et al*., 2005). All three celltypes express TLRs, and the transcripts of 9 TLRs were detected in microglial cell cultures, while astrocytes and oligodendrocytes showed a more restricted expression pattern of TLRs (Bsibsi *et al.*, 2002). Another study demonstrated that microglia are capable of responding to various TLR agonists, further supporting the importance of this cell type as a sensor of pathogens in CNS (Olson *et al.*, 2004). Under basal conditions, the distribution of TLR-2 and TLR-4 expression is limited to the leptomeninges, choroid plexus and the circumventricular

organs (CVO) (Laflamme *et al.*, 2001a and 2001b). These are brain regions where pathogens enter the brain and thus represent strategic locations for pattern recognition receptors. Interestingly, the transcript of TLR-4 is down-regulated after systemic LPS and IL-l-β injection, suggesting transition of the CNS into a hyporesponsive state (Laflamme *et al.*, 2001a). In contrast, the expression of TLR-2 is induced after systemic challenge with LPS, indicating a complex regulation of TLRs (Laflamme *et al.*, 2001b). A deficient immune system may in fact be beneficial for the brain during infection, since immunocompetent mice develop more severe brain inflammation than immunocompromised mice (Merrill *et al.*, 1996).

Despite the presence of active immunity in the CNS there are pathogens that have evolved mechanisms to subvert host defense as part of an invasive strategy. *Neisseria meningitidis* is a neurotropic pathogen with the capacity to cross the BBB and cause meningitis. The endothelial cells of the BBB release a number of proinflammatory cytokines, chemokines and adhesion molecules in response to meningococcal infection (Wells *et al*., 2001). This signaling is independent of TLR-2 and -4 and the precise details of meningococcal interaction with BBB endothelium remain to be determined (Humphries *et al.*, 2005).

Mice lacking the myeloid differentiation factor 88 (MyD88), an essential intracellular signal transducer in TLR signalling, was recently shown to exhibit attenuated brain inflammation after pneumococcal infection (Koedel *et al.*, 2004). Even though these findings suggest an involvement of TLRs in CNS-infection with *Streptococcus pneumoniae,* the precise mechanisms remain elusive. Streptococci may cause sepsis and subsequent invasion of the CNS. However, direct neuroinvasion via the olfactory nerve was recently proposed as a novel route of infection (van Ginkel *et al*., 2003). Afferent nerves are also exploited by *Listeria monocytogenes* to infect the brain. However, IFN-γ stimulated neurons are able to kill *Listeria*, suggesting that neurons possess intrinsic bactericidal activity (Jin *et al.*, 2001).

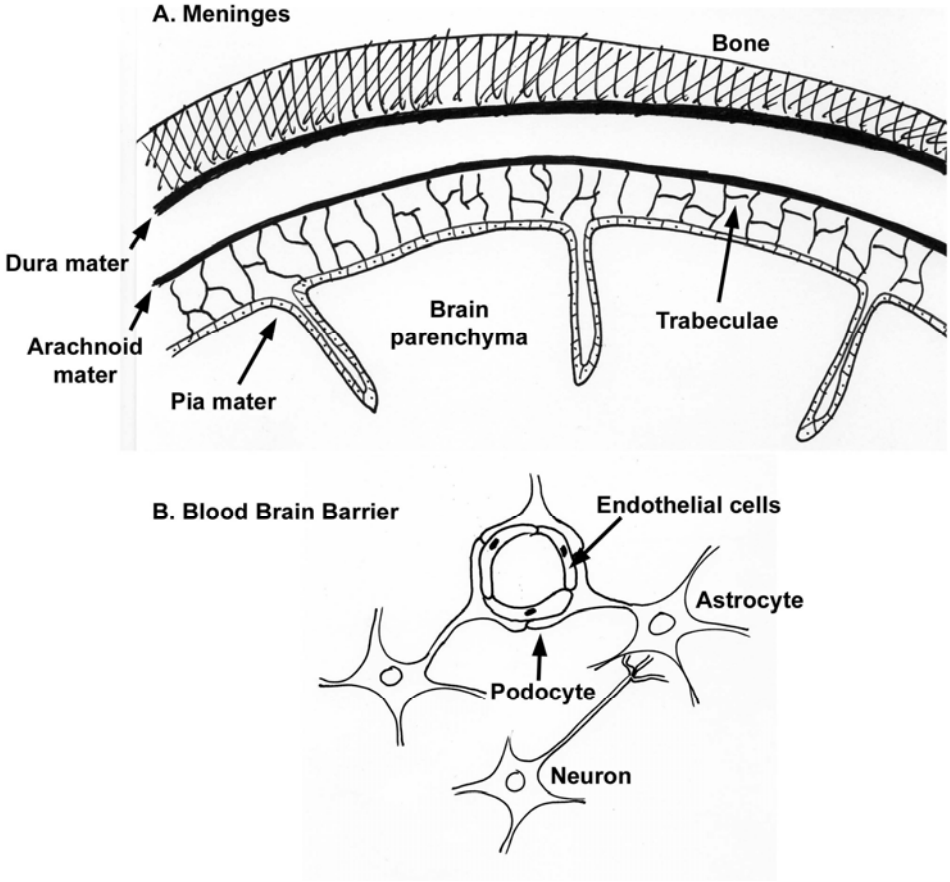

Fig 5. Meninges and the blood brain barrier.

(A) *The meninges protect the brain by forming three separate layers; dura, arachnoid and pia mater. The outermost of these, dura mater, is connected to the bone of the skull, while the arachnoid and pia mater consist of soft tissues (collectively called the leptomeninges). The epithelial-like cells of the leptomeninges are called meningothelial cells. The trabeculae are fibrous structures that connect the arachnoid mater with the pia mater. The trabeculae also contain blood vessels, which are used by bacteria to enter the subarachnoid space (SAS). In the SAS, bacteria multiply and interact with meningothelial cells, which release a number of proinflammatory molecules. This inflammatory reaction is defined as meningitis and may be detrimental to the host.* ***(B)*** *The BBB consists of endothelial cells and foot processes of astrocytes. This arrangement forms a tight barrier that protects the brain against toxins and invasive microbes. However, bacteria, such as* Neisseria meningitidis *and* Streptococcus pneumoniae *may cross the BBB and cause meningitis.*

In fact, IFN-γ has been shown to induce bactericidal activity, especially against intracellular bacteria, in a number of cell types. This activity could be attributed to nitric oxide and 2,3-dioxygenase (IDO, a tryptophan degrading enzyme) in epithelial cells (Hucke *et al.*, 2004), glioblastoma cells (Mackenzie *et al.*, 1998) as well as in choroid plexus cells (Adam *et al.*, 2004) and astrocytes (Oberdorfer *et al.*, 2003). Interestingly, another bactericidal mechanism independent of nitric oxide and IDO has been described in endothelial cells of the BBB. This activity may originate from a

peptide or protein, since it was abrogated by cycloheximide, an inhibitor of protein synthesis (Hoffman *et al.*, 1999).

AMPs are expressed in various cell types of the brain. Bovine β-defensin mRNA is expressed in the meninges, cortex and Purkinje cells of the cerebellum (Stolzenberg *et al.*, 1997). In human, HBD-1 mRNA is constitutively expressed by astrocytes and meningeal fibroblasts, but not in neurons, while HBD-2 can be found in astrocytes after stimulation with LPS (Hao *et al.*, 2001). In addition, HBD-1 mRNA has been detected in the human choroid plexus (Nakayama *et al.*, 1999). Interestingly, many of the recently discovered β-defensins in mice and humans are expressed in reproductive and brain tissues, but their role remains to be elucidated (Maxwell *et al.*, 2003).

In conclusion, the brain has a potent immune system consisting of both BBB endothelial cells and microglia in the brain parenchyma. Even though brain infection is a rare phenomenon, there are microbes with the capacity to circumvent the defenses of the host, causing meningitis or encephalitis. There are several antimicrobial factors present in neural and glial cells, such as nitric oxide and IDO. However, increasing evidence suggest that also AMPs play a role in the defense of the brain against infection. Particularly interesting is the finding that a large number of novel β-defensin genes are actively transcribed in the brain of mice and humans.

Aims

The general aim of this thesis was to isolate antimicrobial peptides from tissue-extracts and to characterize their interactions with pathogenic *Neisseria*, both *in vitro* and *in vivo*.

More specifically the aims were:

I. To isolate and characterize novel antimicrobial compounds from a peptide/protein extract of human colon mucosa.

II. To evaluate the effects of *Neisseria gonorrhoea* on LL-37 expression in human cervical epithelial cells.

III. To isolate and characterize the cathelicidin AMP rCRAMP in rat brain. To evaluate the bactericidal effect of rCRAMP against *Neisseria meningitidis*.

IV. To study the role of the complement regulator CD46 in a transgenic mouse model of *Neisseria meningitidis* infection.

V. To assess the role of the antimicrobial peptide CRAMP in meningococcal infection in mice.

Aspects on methodology

(or how to discriminate between facts and artifacts)

I will here comment on some of the methods I have used in this thesis. I will start with the preparation of peptide/protein extracts and continue with the identification of known and novel peptides, at both the peptide and transcriptional levels. This is followed by methods I have employed to determine the cellular source(s) of peptides. Finally, I will comment on experimental systems to evaluate functional aspects of antimicrobial peptides.

Preparation of peptide/protein extracts

Tissues from the organ of interest are removed and immediately frozen on dry ice, in order to avoid activation of endogenous proteases. Frozen tissues are then ground into small pieces in a mortar with liquid nitrogen and homogenized in 60% acetonitrile with 1% trifluoroacetic acid (TFA). Peptides and proteins are extracted in the same solution at 4°C overnight. The next day, undissolved material is removed by centrifugation and the supernatants are frozen and lyophilized. The extract is then re-dissolved and applied to OASIS-columns in order to enrich for peptides and proteins together with the removal of salts and lipids, which may interfere with subsequent purification procedures. In this protocol, the addition of protease inhibitors is not included, assuming that the low pH caused by the addition of the strong acid TFA (pKa = 0.3) inactivates most of the common proteases. Acid extractions have been extensively utilized to purify peptides and proteins from natural sources (Mutt, 1988).

Peptide/protein extracts have been used for several purposes depending on the hypothesis. When I searched for novel antimicrobial peptides or proteins, extracts were screened for antimicrobial activity using the inhibition zone assay. This is the first step before setting up a purification scheme in order to identify active antimicrobial components of the extract.

Antimicrobial assay (inhibition zone assay)

Thin agarose plates in Luria Bertani (LB) medium containing the test microbe (*B. megaterium* strain Bm11 or *E. coli* strain D21) are prepared. Wells of 3 mm are punched in the plates and 3 µl of the sample are loaded into each well. After an overnight incubation the zone diameters are measured. In some experiments Medium E, a physiological salt medium developed for *E. coli*, is added to the agarose (Vogel *et al.*, 1956). Medium E is known to promote α-helical folding in linear antimicrobial peptides (Johansson *et al*., 1998), such as LL-37, CRAMP and rCRAMP. The

inhibition zone assay is a quick and informative method to analyze the total antimicrobial activity in peptide/protein extracts and chromatographic fractions. However, it is not a good method to compare different extracts or synthetic peptides against each other in killing efficiency. For that purpose, the colony forming unit assay is the method of choice (see below). One major drawback with the inhibition zone assay is that the size of the zones depends on the diffusion-rate of the components in the agarose. The chemical properties of the individual components determine the diffusion-rate, which make comparisons between different extracts difficult. I used this method mainly for screening peptide/protein extracts or chromatographic fractions for the presence of antimicrobial activity.

Identification of AMPs in peptide/protein extracts

To search for known antimicrobial peptides specific antibodies are readily used in immuno dot-blot or Western blot analyses. Large-scale screening can be performed with a dot-blot assay, where 1 µl of each sample is applied to a cellulose membrane. Many samples (n=100) can be analyzed at one time, making it perfect for screening HPLC-fractions. The membrane is incubated with a specific antiserum. The signal is visualized with the ECL detection system. However, the risk for false positives is substantial using dot-blot analysis and therefore the immunoreactive samples should be subjected to Western-blot analysis.

Recent developments in Western-blot protocols have reduced the time for electrophoresis to 30 min when using NU-PAGE gels. Thus, it is possible to perform Western-blot analysis in one day, instead of the usual two-day procedure. Western-blot analysis has the advantage that the size of the immunoreactive peptide or protein is visualized, which reduces the risk of false positives. Moreover, detection of extended or shorter forms of peptides is possible with this method.

If a peptide/protein extract exhibits antimicrobial activity, it is of interest to determine if the activity is peptide-based. The extract is then incubated with proteolytic enzymes, such as proteinase K or pepsin. If the activity is lost, it follows that peptides/proteins are involved. The extract can also be mixed with an antiserum against a specific peptide or protein. If the activity is reduced it is possible to state that a specific peptide is present in the extract and that it contributes to the observed antimicrobial activity. It is, however, important to include a non-specific antibody as a control since the addition of antiserum may decrease the activity in an unspecific manner.

To identify previously unknown antimicrobial peptides or proteins in extracts, the polypeptides have to be isolated to homogeneity. I have used several purification

procedures, including reversed phase, ion exchange and size exclusion chromatographies. The most robust method is reversed phase, where the sample is dissolved in 0.1% TFA and applied to a column (C8 or C18). Hydrophobic molecules bind to the matrix of the column, and can be eluted with a gradient of acetonitrile in 0.1% TFA. Fractions are collected, lyophilized and subjected to further analysis.

To identify polypeptides, mass spectrometry or peptide sequence analysis are utilized. However, the complexity of the material may pose a problem. An informative method to analyze a fraction for its complexity is to apply a small sample of the fraction to a gel, perform electrophoresis and then stain the gel with silver. Silver staining is sensitive and nanogram amounts of polypeptides can be detected. To determine the sequence of the polypeptide, it is necessary to blot the material in the gel to a PVDF membrane, prior to sequence analysis. The Coomassie stained band containing polypeptides in gels is excised with a scalpel and subjected to trypsin degradation and subsequent mass fingerprinting.

Cellular origin of the peptide

After having identified a known or a novel antimicrobial peptide in a peptide/protein extract, it is of interest to determine the cellular source. This is achieved by obtaining intact pieces of the tissue of interest, either frozen or paraffin embedded, and then cut the tissue to thin sections. I have preferred to work with frozen sections. However, the morphology is better preserved with paraffin sections. The sections are incubated with an antiserum specific for the peptide of interest. Visualization is performed with Diaminobenzedene (DAB) or fluorescence, with the latter being more sensitive and also enabling double-staining experiments. Double-staining with specific marker for cell-types, such as neutrophils or epithelial cells, is a good method to investigate co-localization. In combinations with these stainings, good resolution of cells is achieved by confocal microscopy. Furthermore, it is important to perform proper controls. Omitting the primary or the secondary antibody is often used as a control, but is not sufficient to exclude unspecific staining. A more stringent method is absorption of the antibody with its corresponding antigen, which is a control for the particular epitope being recognized by the antiserum. However, epitopes may be shared by unrelated peptides or proteins. Thus, important controls in immunohistochemistry are tissue samples from KO-mice, where specific staining can be separated from non-specific.

Transcriptional level – quantification and localization

One way of analyzing the expression of peptides or proteins is to study the levels of mRNA. This can be performed using Northern blots or PCR based methods. In

Northern blots the total amount of RNA in a tissue or cell culture is separated on a gel and blotted to a membrane. The membrane is then incubated with a specific and complementary probe for the transcript. The drawback of Northern blot analysis is its relative lack of sensitivity and consumption of large amounts of RNA. Therefore, the more sensitive method RT-PCR is commonly utilized to study levels of transcription. In RT-PCR, RNA is isolated and reversly transcribed into cDNA, which is very stable and may be stored for years in the freezer. In addition, cDNA is preferable, since it can be used as template for the subsequent PCR-reaction. Specific primers are used and directed to a defined part of the cDNA (the amplicon), which is amplified during 30, 35 or 40 cycles. The PCR products are then loaded in gels, where the amplicon size is confirmed. Non-specific amplification is common, and it is of importance to confirm the identity of the PCR product by sequencing or Southern blot analysis, using a specific probe for the transcript of interest. RT-PCR is a good method to detect transcription of a particular gene (on/off question). 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 building blocks for new DNA strands are consumed and the amount of synthesized DNA flattens out. In the plateau phase, it is impossible to quantify the number of transcripts (Fig 6).

This problem has partly been solved in real time PCR, where the whole amplification process is recorded. The primers flank a fluorescent probe, which emit light during every cycle. The more transcripts that are produced, the more light is emitted. The intensity of the light is measured “real-time” and is plotted on the y-axis, while the number of cycles is plotted on the x-axis. Thus, it is possible to follow the amplification procedure from the first cycle to the last. A threshold value (Ct) is defined as “the number of cycles it takes to amplify the template reaching a specific light intensity (preferably in the exponential phase). The lower the Ct-value is, the more cDNA was present from the beginning (Fig 6). It is possible to compare Ct-values directly; however the most accurate method is to use a standard curve that is run in parallel with the samples. The standard curve can either be absolute (exact copy number) or relative (a dilution series of cDNA). The easiest and most common method is based on the relative standard curve. This enables quick and easy quantification of gene expression. The result is often expressed as “fold induction” relative to control samples. However, it is not possible to determine absolute copy numbers by using this method.

Since the extraction procedure of RNA may vary between different samples it is important to have an “internal standard”, representing the RNA yield of specific samples. This “internal standard” should preferably be a transcript that is not affected by the experimental conditions. Many of these “internal standards” are designated

"housekeeping genes". The levels of the "gene of interest" are then normalized to the level of the "housekeeping gene". There are many different housekeeping genes available, such as β-actin, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S RNA. There is no obvious choice, since they are all subject to regulation by different external stimuli. It is therefore vital to confirm that the housekeeping gene is not a subject to variation during the experimental conditions. Thus, each experimental system has to be analyzed separately in this respect.

The cellular localization of a transcript is determined by *in situ* hybridization using specific probes complementary to the mRNA encoding the peptide of interest. The probes can be either riboprobes (cRNA) or oligo probes (synthetic DNA oligonucleotides). The method where riboprobes are used is sensitive but more laborious and the probes are easily degraded by RNAses. The oligo probe-method, on the other hand, is not so sensitive but the probes are easy to synthesize and stable over time. Also for *in situ* hybridization, proper controls are of great importance. The best control is of course to use tissue obtained from KO-mice, but the most common control is the sense probe, i.e. the identical series of nucleotides as the mRNA in the tissue. This probe should not anneal to the transcript and should thus give no signal.

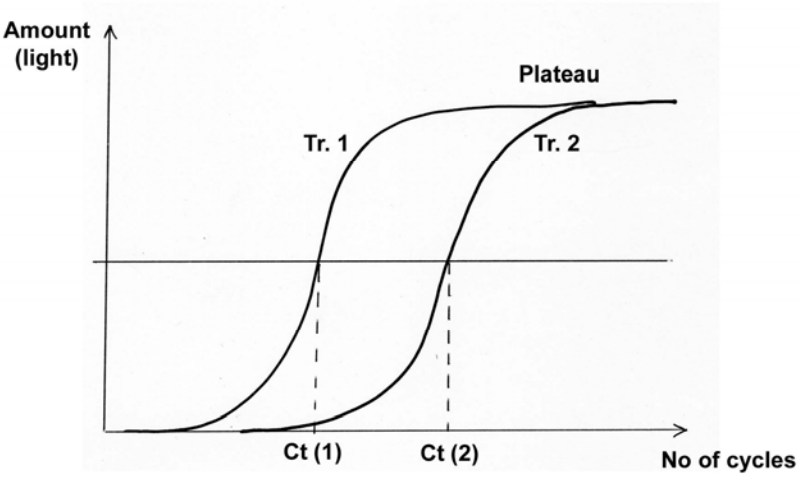

Fig 6. Real Time PCR

Taq-man® Real time PCR is based on the PCR-method, but in addition to primers, a fluorescent probe is included. This probe binds specifically to the amplicon and during each cycle light is emitted, recording the amplification process in "real time". The number of PCR cycles required to reach a certain level of fluorescence is defined as the "threshold cycle number", (Ct-value). The lower the Ct-value is, the more transcripts were present from the beginning. This is illustrated in this figure, where transcript 1 is significantly more abundant than transcript 2, as recorded by their respective Ct-values. Traditional reverse transcriptase based PCR assays (RT-PCR) were performed for a defined number of cycles (30, 35 or 40 cycles). Thus, it was easy to end up in the plateau phase, where quantification could not be performed, due to saturation effects.

Functional role of AMPs

Colony forming unit assay

To obtain detailed information on the killing capacity of antimicrobial peptides for a specific bacterium, a colony forming unit (CFU) assay can be used. This assay is performed in microtiter plates, where serial dilutions of the peptide are mixed with bacteria. This mixture is incubated for 1 or 3 hours (the time has to be determined) and aliquots are plated on agar plates. Next day, the number of surviving colonies is counted. Using this assay, it is possible to calculate the minimal inhibitory concentration (MIC), the minimal bactericidal concentration (MBC) and the effective peptide concentration that kills 50% of the bacteria (EC_{50}). The outcome of the CFU-assay depends on several parameters: bacterial strains, amount of bacteria used, the incubation time and the salt-concentration in the culture medium. Since these experimental conditions differ substantially between different laboratories comparisons of MIC values is complicated. However, despite these shortcomings, the CFU-assay is considered as "the Golden Standard", when determining antimicrobial activity of peptides.

In vitro using cell-lines

The bactericidal effects of cells in culture can be investigated by infecting cells with bacteria. After incubation, the number of recovered bacteria is compared with the initial bacterial counts to create a "growth index". Utilizing specific blocking antibodies or RNA interference, the bactericidal activity of an individual peptide can be assessed. Further, cell-lines are commonly employed for investigation of the expression of an antimicrobial peptide, on both mRNA and peptide levels. The cells can be stimulated with cytokines, growth factors, microbial products, or infected with live bacteria. This cellular treatment is followed by isolation of RNA for transcriptional analysis as well as collection of cell-pellet and supernatant for peptide identification. When using live bacteria, the number of bacteria per cell (multiplicity of infection, MOI) is important to consider. Excess of bacteria may induce cell-death, whereas too few bacteria may not affect the cells at all. In fact, several bacteria – both pathogens and commensal strains – have the capacity to initiate the apoptotic machinery in host cells (Zychlinsky *et al.*, 1997). This has to be taken into account, since apoptosis fundamentally changes the phenotype of cells, and thus may influence the results of transcriptional and peptide analyses. Several recent studies have investigated the effect of LL-37 on host cells (Davidson *et al*., 2004; Braff *et al*., 2005b). Since LL-37 is cytotoxic in high concentrations (Johansson *et al*., 1998), the fraction of dead cells in the culture should be considered. A detailed investigation of

cell viability after LL-37 stimulation was recently published (Steinstraesser *et al*., 2005).

In vivo using KO mice

The function of an antimicrobial peptide may be evaluated *in vivo* using gene deficient mice, i.e. KO-mice. A major problem when working with live animals is the large individual variability. It is therefore important to use sufficient number of animals in each group to be able to draw statistically solid conclusions. The exact number of animals is hard to determine, but 6-10 mice per group is generally considered to yield statistically solid data. In one study of this thesis we have challenged mice with *Neisseria meningitidis*. Even though most mice survived the infection, they were affected by the massive microbial inoculum. The mice developed a "sickness-response", including piloerection, porphyria (red eyes) and autophagy (gnawing on the paws) as well as loss of appetite and weight. All these physiological responses were closely monitored and scored during the course of infection. Before the experiment was started, a "humane end-point" was defined. This occured when the disease score reached a certain level. To avoid unnecessary suffering for the animals, the experiment was then terminated and the mice were euthanized in a humane way (Van Zutphen *et al*., 2001). The problem in infectious challenges of mice is the "read-out" of this *in vivo* assay. The clinical status of the mice is of course of interest, but the judgements are not objective, and the "end point" may be reached before any conclusions can be drawn. A better approach is to monitor the number of bacteria in the blood (CFU-counts). However, a global assessment of the mice would be desired. A combination of the clinical status, bacterial counts and physiological parameters, such as heart rate, blood pressure and inflammatory markers would, in my opinion, enable more informative and ethically acceptable experiments.

Results and discussion

Paper I:

Human colon mucosa contains several antimicrobial peptides and proteins

With the aim of finding novel antimicrobial peptides, a peptide/protein extract was made of human colon mucosa. The extract was active against both Gram-positive and Gram-negative bacteria as well as against the fungus *C. albicans*. By using reversed phase HPLC chromatography, four polypeptides with antimicrobial activity were isolated and characterized. These were ubiquicidin, ECP, PLA_2, and Histone H2B. In addition, we identified LL-37, HNP 1-3 and HBD-1 in this material by using immunological methods and RT-PCR. Taken together, our results are consistent with the notion that the human colon mucosa is protected by a complex mixture of antimicrobial peptides and proteins.

Paper II:

Neisseria gonorrhoeae down-regulates LL-37 in cervical epithelial cells

Neisseria gonorrhoeae is a human pathogen causing the sexually transmitted disease gonorrhea. This bacterium preferentially attaches to and invades epithelial cells of the genital tract. Since these cells express the human cathelicidin LL-37 we wanted to investigate the role of LL-37 during *N. gonorrhoeae* infection. The cervical epithelial cell-line ME 180 was used for this purpose and the expression of LL-37 was confirmed on both peptide and transcriptional levels. Moreover, LL-37 exhibited potent *in vitro* activity against *N. gonorrhoeae*. Interestingly, the levels of LL-37 were down-regulated during the infection, according to quantitative real-time PCR and immunocytochemistry. The down-regulation was most prominent with pathogenic strains of *Neisseria*, while non-pathogenic strains such as *N. lactamica* and *E. coli* BL21 only exhibited moderate effects. Heat-killed bacteria had no impact on the down regulation, stressing the importance of live bacteria. The results in this study suggest that pathogenic *Neisseria* may gain a survival advantage in the female genital tract by down-regulating LL-37 expression.

Paper III:

rCRAMP in rat brain

We raised the hypothesis that the brain, in lack of adaptive immune response, may rely on antimicrobial peptides in the defense against microbes. A peptide/protein

extract of rat brain was active against *E. coli* D21, and the activity was elevated when salt was added to the assay. Antibody depletion experiments showed that the cathelicidin rCRAMP contributed to this activity. Further work could identify rCRAMP in distinct regions of rat brain, both on mRNA and peptide levels. In addition, rCRAMP was active against the neuropathogenic bacterium *Neisseria meningitidis*. Taken together, this study demonstrates that rCRAMP is present in rat brain and may play a role in the protection against neuroinvasive bacteria.

Paper IV:

CD46 transgenic mice are susceptible to meningococcal infection

Neisseria meningitidis is a human pathogen causing sepsis and meningitis. Previous studies have shown that pili of *N. meningitidis* interact with CD46, a human receptor regulating complement activity. Transgenic mice expressing human CD46 were susceptible to meningococcal disease. Both CD46 transgenic and non-transgenic mice developed bacteraemia after intraperitoneal challenge of *N. meningitidis*. However, lethal disease occurred only in CD46 transgenic mice (100% mortality). Bacteria were found in cerebrospinal fluid, in the meninges and in the choroid plexus of CD46 transgenic mice. Intranasal infection of CD46 transgenic mice caused lethal disease in 15% of the animals. Bacteraemia or death occurred only in mice treated with antibiotics prior to the intranasal infection, demonstrating the importance of the normal flora in the protection against this pathogen. In summary, we have found that the CD46 transgenic mouse is a useful model in the study of meningococcal pathogenesis.

Paper V:

CRAMP in the blood brain barrier after meningococcal infection.

The expression of the cathelicidin CRAMP was investigated in mice after meningococcal challenge. By immunohistochemistry, CRAMP was detected in endothelial cells and meninges of infected brains (Fig 7), while non-infected brains did not exhibit any immunoreactivity for CRAMP. In addition, high concentrations of the CRAMP precursor were found in serum of infected mice. Moreover, in a colony forming unit (CFU) assay, the synthetic CRAMP peptide killed *N. meningitidis*. With these data in mind, we hypothesized that CRAMP is involved in the protection against meningococcal infection. CRAMP-KO mice were therefore infected with *N. meningitidis* intraperitoneally. This resulted in significantly higher bacterial counts in blood, liver and spleen 6 hours post-infection compared to wild type (wt) control mice. Of the CRAMP-KO mice, 12 % (3/25) died as a result of the infection, while no

wt mice died. However, both wt mice and the surviving CRAMP-KO mice exhibited transient bacteraemia, but after 48 hours of infection no bacteria were present in the blood. Interestingly, whole blood and serum isolated from KO-mice exhibited decreased capacity to inhibit meningococcal growth. In conclusion, we have shown that CRAMP contributes to host defense early in meningococcal infection.

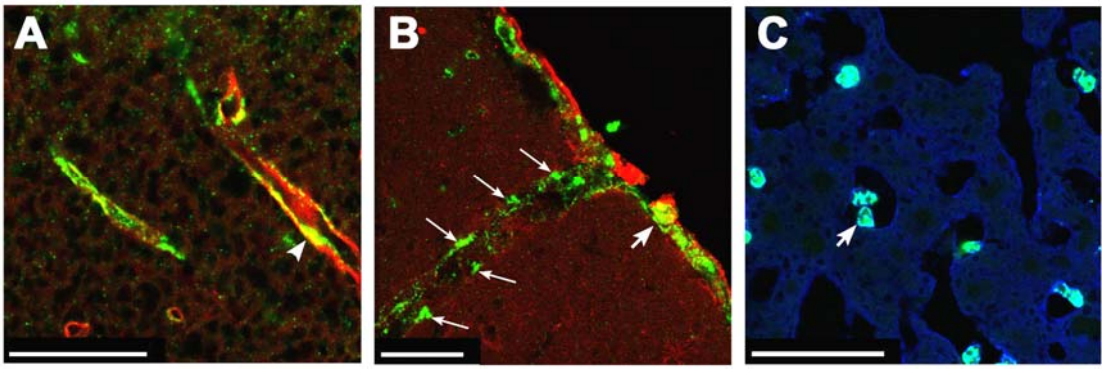

Fig 7. Immunohistochemical detection of CRAMP in brain and liver after meningococcal infection.

(A) CRAMP-like immunoreactivity (LI) (green) was found to be co-localized with CD31 (red), a marker for brain endothelial cells (arrowhead). **(B)** CRAMP-LI was found in meningeal cells (green, thin arrows) as well as in a neutrophil (red, arrow). **(C)** In the liver, CRAMP-LI (green) was found to be co-localized with a marker for neutrophils (arrow), and no staining was detected in endothelial cells. White bar, 50 μm.

Discussion, paper I and II

Antimicrobial peptides at epithelial surfaces of colon and cervix

In paper I, the human colon mucosa was in focus. Since this organ contains 2 kilograms of commensal bacteria there must exist potent mechanism(s) that control bacterial growth and prevent bacteraemia. With this in mind, we hypothesized that the human colon mucosa contains antimicrobial peptides, contributing to the defense against bacteria. We could isolate and characterize several polypeptides with antimicrobial activity. Ubiquicidin is a conserved C-terminal fragment of a 40S ribosomal protein and has previously been isolated from mouse macrophages on the basis of its bactericidal activity (Hiemstra *et al.*, 1999). The present study is the first where ubiquicidin is isolated from human tissue. Histones represent an intracellular group of proteins with antimicrobial activity (Skarnes *et al.*, 1957). The physiological role of these non-secreted proteins in the defense against bacteria is disputed. Recently, it was proposed that the colon keep these molecules inside epithelial cells as a latent intracellular effector (Howell *et al.*, 2003). In addition, release of histone H1 has been proposed to occur during apoptosis (Rose *et al.*, 1998). Since many microorganisms induce apoptosis in epithelial cells (Kim *et al.*, 1998), the release of

intracellular bactericidal compounds may constitute an additional defense mechanism against bacteria (Zychlinsky *et al.*, 1997).

In the human colon mucosa there are resident immune cells present. Therefore it is possible that the isolated peptides and proteins originate from eosinophils, neutrophils and macrophages. In fact, ECP is considered to be a marker for eosinophils (Carlson *et al.*, 1999) and HNP 1-3 are abundant in neutrophils, whereas colon epithelial cells constitutively express LL-37 and HBD-1. In conclusion, our findings suggest that a complex mixture of peptides and proteins, originating from epithelial or immune cells, protects the colon mucosa. Combined, these peptides and proteins constitute a functional barrier with the capacity to kill invading microbes.

This conclusion probably applies equally well to the female genital tract, which share many immunological traits with colon. They both host a normal flora without any signs of inflammation, while retaining the capacity to sense and respond to pathogens (Quayle, 2002). In paper II, we investigated the effects of gonococcal infection on the expression of LL-37 in cervical epithelial cells. Interestingly, we observed a prominent down-regulation of LL-37 transcription, which was more pronounced after infection with pathogenic strains. Invasive bacteria must avoid innate defenses in order to cause disease, and one way to accomplish this strategy may be interference with antimicrobial peptide expression. In the colon, *Shigella* down regulates LL-37 (Islam *et al.*, 2001) and in the small intestine pathogenic *Salmonella* suppresses transcription of Paneth cell defensins (Salzman *et al.,* 2003a). Our results indicate that gonococci also down-regulate an innate effector molecule, such as LL-37, as a potential immune escape mechanism. In colon epithelial cells, butyrate is a potent inducer of LL-37 expression (Hase *et al*., 2002; Schauber *et al.,* 2003). We found that also cervical epithelial cells responded to butyrate stimulation by induction of LL-37 expression. After 24 hours of butyrate stimulation the levels of LL-37 were increased by 10-15-fold compared to control cells. Interestingly, even after this incubation with butyrate, gonococcal infection was able to down-regulate LL-37 expression, indicating that gonococci interfere with butyrate signaling. Detailed molecular knowledge of the mechanism involved could lead to novel treatments of gonococcal infection.

The neisserial factor responsible for the down-regulation remains unknown. Our data suggest that the down regulatory effect was abrogated when the bacteria were heat-killed. This implies the involvement of either live bacteria or a heat-sensitive factor. It excludes the involvement of LOS, which is heat-stable. Consequently, a recent study has shown that gonococci activate host cells independently of TLR-4 (Fichorova *et al.*, 2002). Our studies further suggest that the down-regulation is independent of pili, since non-piliated strains exhibited the same effect. Previous work has shown that

non-piliated strains induce transcriptional changes in ME 180-cells (Plant *et al.*, 2004). Recently, secreted proteins from meningococci were shown to up-regulate several proinflammatory genes in host cells (Robinson *et al.*, 2004). It is possible that the down-regulation of LL-37 in our study, in fact, is mediated via secreted gonococcal proteins. Future studies, addressing this particular issue, would be informative.

Discussion, paper III, IV and V

Antimicrobial peptides in the brain and meningococcal infection

The brain is an immunopriviliged organ that is protected against immunecells, toxins and pathogens by the blood-brain barrier. Previous work has identified defensins in the brain of different mammalian species, but the possible role of cathelicidin antimicrobial peptides in the brain has not been investigated. A peptide/protein extract was made of rat brains and found to be active against *E. coli*. Interestingly, the activity increased when salt was added, which could indicate the presence of α-helical antimicrobial peptides. Western blot analysis using rCRAMP specific antiserum, demonstrated that rCRAMP was present in the whole brain extract. When different brain regions were analyzed, the peptide and the corresponding transcript were found in olfactory bulb, medulla, cerebellum, and spinal cord. rCRAMP-like immunoreactivity was detected in olfactory bulb, cerebellum and spinal cord by immunohistochemistry. In addition, detailed analysis, using primary cell cultures derived from rat brain revealed that the gene also was expressed in hippocampus and striatum. The cellular localization in neurons of the olfactory bulb and spinal cord could be in line with a role of rCRAMP in antimicrobial defense of the brain. The massive bacterial load in the nasal cavity, suggest that there are potent innate immune mechanisms present. The olfactory nerve is used for pathogens to gain access to the brain (van Ginkel *et al*., 2003). Recently, NPY was suggested to play a role in the protection of nasal epithelium and the olfactory bulb against microbial invasion (Brogden *et al.*, 2005). However, the innate immunity of the nasal tract is not fully elucidated, and it is possible that rCRAMP plays a role in host defense of this anatomical region.

In the spinal cord, viruses and bacteria may exploit sensory nerves to invade the CNS. It is therefore possible that ganglia and nerves possess intrinsic microbicidal mechanisms as self-defense. Indeed, IFN-γ treated neurons were able to kill *Listeria monocytogenes,* via an unknown mechanism (Jin *et al.,* 2001). We propose that rCRAMP may be a possible mediator of such an effect. However, this speculative statement remains to be confirmed.

The finding of rCRAMP in cerebellum is not in line with a direct microbicidal effect, but novel and unexpected functions of this peptide may be considered. Furthermore, rCRAMP was active against the neuropathogenic bacterium *Neisseria meningitidis*. To our knowledge, this is the first study demonstrating that an antimicrobial peptide can kill encapsulated bacteria. Taken together, these data demonstrate that the cathelicidin rCRAMP is expressed in rat brain and may play a role in the protection against neuroinvasive bacteria.

Neisseria meningitidis only infects humans and the reason for this preference is not completely elucidated. One explanation may involve the lack of host receptors for *N. meningitidis* in mice. Previous work has suggested that the main receptor for attachment of *N. meningitidis* to human epithelial cells is CD46. In the present study, we have investigated the role of CD46 in meningococcal disease using transgenic mice expressing CD46. Interestingly, these mice were susceptible to meningococcal infection, when infected intraperitoneally. Bacteraemia was evident in both transgenic and wild type strains. However, crossing of the blood brain barrier occurred only in transgenic mice. Non-piliated strains were slightly more virulent than pilated strains, indicating that pili may not be the main virulence determinant when inoculating via the i.p. route. However, when infecting mice via the intranasal route, only piliated strains caused disease. Intranasal infection required pre-treatment with antibiotics and resulted in low mortality (15%), despite inoculation with high doses of bacteria. This suggests that there is a potent innate immune system present in the upper respiratory tract. Taken together, these data support the crucial role of CD46 in meningococcal pathogenesis. However, the detailed interactions between pili and CD46 are not established, and recently *Neisseria* was proposed to bind to additional receptor(s) (Kirchner *et al.*, 2005a).

Since we found the rat cathelicidin rCRAMP in the brain (Paper III), and noticed potent killing of *Neisseria meningitidis*, we wanted to extend these studies to include meningococcal challenge *in vivo*. We decided to use mice for this purpose, and CRAMP-KO mice were generously provided by Dr. Richard L. Gallo, UCSD, San Diego, USA. First we infected C57/B6 mice with meningococci via the intraperitoneal route. By immunohistochemistry we observed induction of CRAMP in endothelial cells of the BBB and in meninges. Immunoreactivity for CRAMP could not be detected in the corresponding parts of non-infected brains. This was in contrast to the rat brain, where rCRAMP was present in non-infected animals. This discrepancy could be due to a difference in antibody sensitivity, or that rat and mouse are different in this respect. In addition, we never infected rats with meningococci, and it is possible that endothelial and meningeal cells of the rat brains also have the capacity to upregulate cathelicidin expression. The finding of CRAMP at strategic locations in

the brain after infection is consistent with the hypothesis that CRAMP protects the brain against invasive pathogens. However, cerebrospinal fluid from both CRAMP-KO mice and wild type 129/SVJ mice contained meningococci after meningococcal challenge via the i.p. route. This was in contrast to C57/B6 mice, which did not develop meningitis, indicating a strain difference in susceptibility to meningococcal disease. The fact that mouse strains exhibit different susceptibility to infectious disease is previously known (Lam-Yuk-Tseung *et al.*, 2003). Our findings suggest that there are additional factor(s) or mechanism(s), apart from CRAMP, that determine bacterial crossing of the BBB. During the experiments, 12 % (3/25) of the CRAMP-KO mice died, while all wt mice survived. Bacterial counts in the blood revealed an increased bacterial growth in CRAMP-KO blood, both *in vivo* and *ex vivo*, most prominent at early time points (6 hours).

To summarize papers III, IV and V, we have found rCRAMP in rat brain and this peptide could kill *N. meningitidis*, despite the presence of bacterial capsule. rCRAMP was expressed in strategic locations, such as olfactory bulb and spinal cord, and may thus play a role in the defense against invading pathogens. Meningococcal infection was studied in mice, and we could show that CD46 is a crucial protein for development of meningococcal disease in mice. However, the detailed interaction of this protein and *N. meningitidis* remains to be determined. Mouse CRAMP was found to be induced in the BBB and meninges after meningococcal infection, although CRAMP does not appear to be the main determinant for bacterial crossing of the BBB. However, CRAMP contributes to innate immunity against meningococci by reducing bacterial growth in blood, especially in early stage of the infection.

Conclusions

From the results presented in this thesis the following specific conclusions can be drawn:

I. A complex mixture of AMPs and proteins is present in the human colon mucosa, consistent with a protective role against invading pathogens.

II. *Neisseria gonorrhoea* down-regulates the expression of the human cathelicidin LL-37 in cervical epithelial cells. This may correspond to an invasive strategy utilized by pathogenic *Neisseria.*

III. The rat cathelicidin rCRAMP is present in the central nervous system, with a distinct regional distribution. The rCRAMP peptide exhibits bactericidal activity against the neuroinvasive pathogen *Neisseria meningitidis*.

IV. CD46 transgenic mice are susceptible to meningococcal infection.

V. The mouse cathelicidin CRAMP is induced in the BBB and meninges after meningococcal infection. In addition, CRAMP inhibits bacterial growth in blood *ex vivo* and *in vivo*.

Future perspectives

Bacterial resistance against conventional antibiotics is an emerging clinical problem (Novak *et al*., 1999; Zetola *et al.,* 2005), making the development of novel antibiotics a highly warranted issue. Therefore, the search for novel AMPs both in humans, and in other species, is of great interest. Antimicrobial peptides isolated from biological material may be used as antibiotics *per se,* or serve as templates for custom designed peptides. However, peptides are not the optimal drug, as they are expensive to synthesize and difficult to deliver. A better way would be to induce the expression of endogenous antimicrobial peptides by small exogenous drug-like compounds, preferably meeting the pharmacokinetic requirements outlined by Lipinski and Hopkins (2004). By developing tissue-specific inducers, the treatment of infection in a single organ would be possible. This strategy would enable high levels of antimicrobial peptides at the site of infection, while systemic concentration would remain low, thus reducing the risk of adverse effects. Consequently, it is important to continue the search for novel antimicrobial peptides by large-scale isolation approaches using biological material. In addition, a detailed understanding of antimicrobial peptide expression in various tissues will facilitate the development of exogenous inducers.

Our finding that *Neisseria* is able to down-regulate LL-37 expression in cervical epithelial cells may have several interesting implications. The identification of the bacterial factor can lead to the development of blocking drugs, which can abrogate bacterial manipulation with host defense. On the host side, the identification of the receptor or signal transduction pathways can lead to novel treatment strategies. In addition, it would be interesting to screen epithelial cell lines, such as ME-180, for inducers of LL-37 and other antimicrobial peptides. Isoleucine was identified as a potent inducer of HBD-2 in epithelial cells (Fehlbaum *et al.,* 2000). The fatty acid butyrate has been shown to up-regulate LL-37 expression in colonic epithelial cells (Hase *et al*., 2002; Schauber *et al.,* 2003). In addition, Vitamin D stimulates LL-37 expression in keratinocytes of the skin (Wang *et al.,* 2004; Weber *et al*., 2005). These compounds are easy to obtain and cheap and the possibility should be considered that they might be used as novel treatments against infectious disease. This would be especially attractive in the developing world, where conventional antibiotics may not be available.

A main finding in this thesis is that AMPs of the cathelicidin family are present in the brain. The results presented here may serve as a starting point for future studies on the role of antimicrobial peptides in meningococcal disease. Of great interest would be to investigate the situation in humans. Are invasive clinical isolates from blood or CSF

more resistant to LL-37 than non-invasive isolates from the upper respiratory tract? Do individuals with a history of meningococcal disease have mutations in the gene encoding LL-37 or exhibit decreased levels of LL-37 in neutrophils or epithelia? A detailed understanding of the mechanism behind CRAMP induction in the BBB can possibly open up for novel treatments against meningococcal disease as well as other neuropathogenic microbes. Further investigations using *in vitro* systems of the BBB can facilitate such studies.

The paradox of scientific discovery:

"If you know just what you are looking for, finding it can hardly count as a discovery, since it was fully anticipated. But if, on the other hand, you have no notion of what you are looking for, you cannot know when you have found it."

Steve Shapin, Harvard University

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