Actions of Antimicrobial Peptides and Bacterial Components in Inflammation

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Abstract

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By using computer simulation we identified an active domain of LL-37, a 21 amino acid fragment, GKE, displaying similar antimicrobial and LPS-binding activity in vitro as native LL-37 but less toxic and therefore holding promise as a template for the development of peptide antibiotics for treating sepsis.

Sepsis due to Gram-positive bacteria is becoming increasingly prevalent. The Gram-positive bacterium Streptococcus pyogenes, carrying a surface protein named M1 being fundamental for its virulence, is the major cause of severe streptococcal infections like streptococcal toxic shock syndrome and necrotizing fasciitis. We found that M1 protein is able to induce vascular nitric oxide (NO) production, which in turn relaxes smooth muscle cells and could thereby contribute to the severe hypotension seen in septic patients. This we confirmed by showing that M1 protein caused hyporesponsiveness to the vasoconstrictor, phenylephrine, in rat aorta.

Bacterial compounds are able to activate Toll-like receptors generating an inflammatory response. Experiments using wild type and knockout mice revealed that M1 protein is able to attach to both TLR2 and TLR4 (TLR) in mice, only activating the latter. M1 protein only attached to TLR2 in human blood vessels.

LL-37 possesses immunomodulatory effects. In order to explore potential modulatory effects of GKE on vascular nitric oxide production we used the well identified proinflammatory compounds interleukin-1β (IL-1β), M1 protein from Streptococcus pyogenes and lipoteichoic acid (LTA). The results showed that GKE seems to have complex modulatory effects on vascular nitric oxide production depending on the inflammatory compound used.

Key words: antimicrobial peptides, LL-37, sepsis, M1 protein, S. pyogenes, Toll-like receptors, nitric oxide, phenylephrine

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Thorgerdur Sigurdardottir M.D.

Lund 2009

To Sveinbjörn, Einar, Helga

and

My beloved parents

CONTENTS

ABSTRACT

Antimicrobial peptides are evolutionally ancient parts of the innate immune system and their primary role is to protect us from infections. The human cathelicidin-derived antimicrobial peptide, LL-37, not only possesses broad spectrum antimicrobial activities but is also able to bind and neutralize bacterial lipopolysaccharide (LPS), an important trigger of the widespread inflammatory response contributing to septic shock. LL-37 has been studied as an alternative to conventional antibiotics but clinical trials have been hampered by indications of its toxic effect on mammalian cells and evidence that its antimicrobial effects are inhibited by serum. It has been proposed that the cytotoxicity of LL-37 is due to hydrophobic amino acids. We were able, by removing hydrophobic amino acids from the N-terminal of LL-37, to generate less cytotoxic peptides with retained antimicrobial and LPS–neutralizing actions in serum.

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vascular NO production in rat aorta. GKE at low concentration inhibited IL-1β-induced NO production, but synergistically increased it at higher concentrations. GKE did not affect the M1 protein induced NO production while GKE inhibited LTA induced NO production. Thus, GKE seems to have complex modulatory effects on vascular nitric oxide production depending on the inflammatory compound used.

ABBREVATIONS

CF	Carboxyfluorescein
E. Coli	Escherichia coli
FPRL-1	Formyl peptide receptor like-1
G-CSF	Granulocyte colony stimulating factor
GAS	Group A *Streptococcus*
IL-1β	Interleukin-1β
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
L-NAME	L-N-nitroarginine methyl ester
L-NMMA	L-N-monomethylarginine
MEC	Minimal effective concentration
NO	Nitric oxide
NOS	Nitric oxide synthase
PAMP	Pathogen associated microbial pattern
PE	Phenylephrine
PMN	Polymorphonuclear neutrophils
STSS	Streptococcal toxic shock syndrome
SIRS	Systemic inflammatory response syndrome
TLR	Toll-like receptor

LIST OF PAPERS

The present thesis is based on the following papers

I Christina D. Ciornei, Thorgerdur Sigurdardottir, Arthur Schmidtchen, Mikael Bodelsson: Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity and inhibition by serum of analogs of human cathelicidin LL-37. *Antimicrobial agents and chemotherapy* 2005; 49(7): 2845-2850.

II Thorgerdur Sigurdardottir, Pia Andersson, Mina Davoudi, Martin Malmsten, Arthur Schmidtchen, Mikael Bodelsson: In Silico identification and biological evaluation of antimicrobial peptides based on human cathelicidin LL-37. *Antimicrobial agents and chemotherapy* 2006; 50(9): 2983-2989.

III Thorgerdur Sigurdardottir, Sigurbjörg Rutardottir, Matthias Mörgelin, Heiko Herwald, Viveka Björck, Mikael Bodelsson: M1 protein from *Streptococcus pyogenes* induces nitric oxide-mediated vascular hyporesponsiveness to phenylephrine: Involvement of toll-like receptor activation. Submitted.

IV Thorgerdur Sigurdardottir, Johan Törnebrandt, Sigurbjörg Rutardottir, Mikael Bodelsson: The LL-37 derived peptide GKE has modulatory effects on vascular nitric oxide production. Manuscript

BACKGROUND

Definition of sepsis and epidemiology

Sepsis is a life threatening and complex clinical syndrome that affects all ages (1). Sepsis occurs when physical injury or immunosuppression damages the host's normal barrier against microbes resulting in a profound inflammatory response. Evident or a suspected infection, in addition to two or more of the systemic inflammatory response (SIRS) criteria are required for diagnosis (Table 1,(2)). Severe sepsis (sepsis accompanied with hypoperfusion or dysfunction of at least one organ system) and septic shock (sepsis accompanied by need for vasopressor despite adequate fluid resuscitation) carry high mortality rates of 30% and 40-70%, respectively, and are the most common causes of death in intensive care units in the western world (3, 4). Sepsis can be caused by Gram-negative and Gram-positive bacteria, viruses and fungi. Bacterial toxins or constituents can also evoke a sepsis-like syndrome. Though epidemiological studies of sepsis through the last decades have been quite difficult to interpret due to lack of a uniform definition of the disease, there seems to be a worldwide increase in the incidence of sepsis; from 82 cases per 100,000 population in 1979 to 240 per 100,000 in 2000 in USA, but the overall mortality rate has been declining during the same period from 27.8% to 17.9% (5). Despite improved survival rates, three times more people are dying each year from sepsis due to increased incidence of the disease (5, 6).

Interestingly, over the past two decades, there has been a shift from Gram-negative bacteria as the leading cause of sepsis towards Gram-positive bacteria, now accounting for over 50% of the cases. This shift could, at least partly, be explained by marketing of efficacious Gram-negative antibiotics, such as third generation cephalosporins as well as quinolones and the increased use of central venous catheters in the eighties. Also of notice, the mortality related to Gram-negative organisms has decreased over the years, whereas mortality has remained static for sepsis induced by Gram-positive bacteria (5). The incidence of severe invasive group A streptococcal infections (Gram-positive) is believed to have reemerged during the past 10-20 years (7).

The rate of candidemia (sepsis due to fungal organisms of *Candida* species) has increased by over 200 %, the last decades (5). Risk factors for candidemia include prior surgery, acute renal failure, and parenteral nutrition, in addition to multiple or prolonged antimicrobial treatment, neutropenia and central venous catheters (8). *Candida albicans* is a predominant species involved in both adult

and infantile candidemia while *C. parapsilosis* is an increasing cause in neonatal acquired fungal sepsis (9).

Table 1. Systemic inflammatory response syndrome (SIRS) (2).

Diagnosis includes two or more of the following criteria:
(1) temperature > 38 º C or < 36 ºC
(2) heart rate > 90 beats per minute
(3) respiratory rate > 20 breaths per minute or $PaCO_2$ < 32 mm Hg
(4) white blood cell count > 12,000 / mm^3 or < 4,000/ mm^3 or > 10% immature (band) forms.

Innate and adaptive immunity

The innate and adaptive immune systems are responsible for the host's defence against infections. Being in the first line, innate immunity includes phagocytotic cells like neutrophils, monocytes and macrophages, which without prior exposure and by the aid of enzymes and antimicrobial peptides rapidly try to battle the invading pathogens (10). These immune cells have surface bound pattern recognition receptors, e.g. Toll-like receptors (TLR), which bind to pathogen associated microbial patterns (PAMPs) shed from or present on the surface of the invading microbes. PAMPs include lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycan and lipoteichoic acids (LTA) from Gram-positive bacteria, flagellin and viral RNA (11, 12). Vascular endothelial cells are among the first cells in the body that come into direct contact with circulating bacterial compounds. It is thus not surprising that endothelial cells also have pattern recognition receptors (13). TLR family members are expressed differentially among immune cells. Toll was originally described in the fruit fly *Drosophilia* as a type 1 transmembrane receptor controlling dorsal-ventral polarity during embryogenesis. Toll mutant fruit fly embryos failed to develop ventral and dorsal cell types, which the German scientists found very cool and funny, hence the name Toll (toll = funny in German) (14). I am not sure that I agree with the Germans there! Mammalian homologs to *Drosophilia* Toll are thus named TLR. To date, 10 human TLR (TLR1-TLR10) have been described, TLR2 and TLR4 being the

far most intensively studied mammalianToll (12). TLR act as microbial sensors and central evidence for their importance comes from models of infection in TLR-deficient mice (15, 16). Previous studies have revealed that LPS primarily binds to TLR4 and Gram-positive PAMPs to TLR2 (11, 15-17). When binding occurs, intracellular signalling pathways are activated, including release of NF-κβ, leading to production of various proinflammatory cytokines and nitric oxide (NO), a powerful vasodilator in sepsis-induced hypotension (18, 19).

While innate immunity acts broadly and quite unspecifically, the adaptive immune system manifests a high specificity for its target antigens. The pathogen must, however, first be recognized by the innate immune system, which then activates the adaptive immune system Thus, synergy between the two is essential in order to get a fully effective immune response (20). Innate immunity reacts immediately upon infection but the adaptive response is primarily based on the antigen-specific receptor expressed on T- and B-lymphocytes and becomes prominent several days after infection, when antigen-specific T and B cells have undergone clonal expansion (21).

Antimicrobial peptides

Antimicrobial peptides are the body's own antibiotics protecting us from the invasion of microbes and, if infection occurs, contributing to a first defence (22). They can naturally be found in the interface between an organism and the environment such as the human dermis, epithelium of airways and gut, seminal and vaginal fluids, breast milk and the cavernous vernix of the newborn (23).

Many antimicrobial peptides share the similar features of hydrophobic and hydrophilic amino acid residues arranged in an amphipathic alfa-helix as well as positive net charge (23). In this way, antimicrobial peptides can bind to bacteria, not only with hydrophobic but also through electrostatic interactions (24). The exact ways in which antimicrobial peptides kill or inhibit the growth microbes is not completely characterized, but most of them seem to exert their antimicrobial activity by disrupting bacterial, fungal and viral membranes causing lysis(25). The specific affection of antimicrobial peptides for microbes compared to mammalian cells is partly due to the fact that the net charge of biomembranes is largely based on its phospolipid stoichiometry and architecture. Bacterial biomembranes tend to have a highly electronegative composition of their phospholipids whereas mammalial cell membranes generally have a neutral net charge shielding them from the actions of the

cationic antimicrobial peptides (22, 26). Other mechanisms for killing of microbes by antimicrobial peptides such as activation of proteolytic enzymes, have also been proposed (24).

The human cathelicidin antimicrobial peptide, LL-37, was discovered independently by three groups in 1995 (27-29). It is mainly stored in granules of mammalian neutrophils and is released when neutrophils get activated at the site of inflammation and infection (30). LL-37 is also expressed in various epithelial cells and its expression can, at least experimentally, be further induced by growth factors and vitamin D (31-33). LL-37, which consists of 37 amino acids, displays an amphipathic α-helical structure and carries a positive net charge of +6 at physiological pH. LL-37 not only possesses extensive antibacterial properties against Gram-positive and Gram-negative bacteria as well as fungi and enveloped viruses, but is also active against microbes resistant to conventional antibiotics such as methicillin-resistant *Staphylococcus aureus* (30). Another important property of LL-37 is its ability to bind and neutralize lipopolysaccharides (LPS) from the cell wall of Gram-negative bacteria, one of the major activator of the inflammatory response in Gram-negative sepsis (29, 34). Furthermore, LL-37 attracts neutrophils, monocytes and T-lymphocytes via activation of the formyl peptide receptor like-1, FPRL1 (35).

Few if any diseases have been directly linked to the lack of LL-37 but the rare disease Kostmann syndrome is worth mentioning. The syndrome, also known as severe congenital neutropenia, is characterized by low levels of neutrophils. In the past, the affected children usually died in early childhood from severe bacterial infections. Although granulocyte-colony stimulation factor (G-CSF) can today normalize the neutrophil count, these patients still suffer from periodontal diseases. The saliva from these patients containes no LL-37 but whether the deficiency of LL-37 is directly linked to the formation of periodontal disease remains to be determined (36, 37).

The combination of broad spectrum antimicrobial and LPS-binding properties makes LL-37 an attractive candidate for adjuvant treatment of sepsis, especially today when resistance towards conventional antibiotics is becoming an increasing problem (38). Unfortunately, native LL-37, like many other antimicrobial peptides, has been shown to be toxic to human eukaryotic cells in concentrations slightly above those needed for its antimicrobial activity (34, 39). Thus, the therapeutic window of the endogenous “antibiotic” LL-37 seems to be rather narrow. It has been proposed that the cytotoxicity is due to the hydrophobic properties of LL-37 (40). The cytotoxic effects of LL-37 liberated

into the circulation is inhibited by its binding to plasma proteins, e.g. apolipoprotein A-1. The binding unfortunately also inhibits the antimicrobial activities of the peptide, rendering naturally occurring LL-37 ineffective as a future peptide-based antibiotic for treating sepsis (39, 41, 42). Thus, one of the goals of this thesis was to find a peptide based on LL-37, which was less toxic and was active in plasma.

M1 protein from Streptococcus pyogenes

Streptococcus pyogenes is a Gram-positive bacterium belonging to the Lancefield streptococci group A (GAS) (43). It usually causes mild infections like bacterial pharyngitis and impetigo and, in fact, streptococcal sore throat is the most common bacterial infection in childhood, of which group A is responsible for the great majority. Scarlet fever results from streptococcal strains carrying pyrogenic exotoxins and is usually associated with pharyngeal infections. Streptococcal pharyngitis is treated with antibiotics not because of the infection per se, which is usually self-limited, but in order to minimize the risk for the late, but serious, nonsuppurative complications glomerulonephritis and rheumatic fever. Importantly, invasive infections due to *S. pyogenes* seem to be increasingly prevalent around the world, though it is not sure that this is a truly significant increase or due to increased interest and alertness regarding this pathogen (44-46). These infections include streptococcal toxic shock syndrome (STSS) and necrotizing fasciitis, carrying mortality rates of 45-85 % despite modern antibiotics and intensive care therapy (46). The fact that STSS is able to affect young and previously healthy individuals is of great concern. All invasive *S. pyogenes* strains carry α-helical coiled coiled M proteins on their surface, being fundamental for their virulence (45, 47). Strains rich in M protein rapidly multiply in human blood where they by binding to various host proteins block opsonisation by antibodies and complement thereby preventing phagocytosis of the bacteria by neutrophils (45, 48). About 150 different M proteins have yet been identified, and are named in numerical order after time of discovery, M1 – M150 (49). *S. pyogenes* carrying M1 protein has been globally disseminated during the last decades and is the most prevalent strain isolated from patients with severe invasive infections (44-46, 50). M1 has also been reported to be an independent predictor of death (7).

It has been shown that M1 proteins induce interleukin (IL)-1β, IL-6 and tumor necrosis factor-α expression in human peripheral blood monocytes (51). It was demonstrated that M1 protein binds to TLR2 on these cells. Recently, soluble

M1 protein has been identified as a superantigen, contributing to the hyperinflammatory respsonse seen in severe invasive streptococcal infections (52). The finding that M1 protein interacts with TLR2 on monocytes suggests that it may be an important PAMP. A TLR-mediated effect by M1 protein on the vasculature is not known but if so could contribute to the severe circulatory dysfunction accompanying invasive streptococcal infections.

The role of nitric oxide in sepsis

Nitric oxide (NO) is a highly diffusible lipophilic gas with a halflife of 6-10 seconds in aqueous environments because of its rapid oxidation to nitrite and nitrate. In 1980, elevated urinary nitrate levels were reported in humans with diarrhoea and fever, and subsequently LPS-challenged rats were shown to have urinary nitrate levels in correlation with their degree of fever (53, 54). When the role of NO in vasodilatation was discovered, the interest in NO as one of the causative agents of septic shock escalated further (18, 55, 56).

NO is primarily synthesized by nitric oxide synthase (NOS) family proteins according to the equation: 1 L-arginine + 1 NADPH + 2 O_2 = 1 L-citrullin + NO + 2 H_2O (Fig 1). Up to date, three different NOS have been identified named neuronal NOS (nNOS*),* endothelial NOS (eNOS) and inducible NOS (iNOS). NO interacts with the enzyme soluble guanylate synthase. Binding of NO to this enzyme leads to the production of the second messenger molecule, cGMP, resulting in NO-dependent relaxation of vascular smooth muscle. In healthy states, NO is continuously produced and plays an important role in controlling normal vascular tone. (19)

Stimulation of TLR leads to a complex intracellular cascade triggering activation and production of cytokines as well as nitric oxide. In severe infections the cytokine release and NO formation can result in systemic vasodilation, diminished myocardial contractility and activation of the coagulation cascade, all hallmarks of sepsis (19). The crucial role of NO in LPS-induced SIRS was established in iNOS knockout mice showing only minimal symptoms after LPS injection when compared to wild type mice having severe hypotension and 60% mortality (57). In vivo studies with septic rodents treated with NOS inhibitors were promising and the expectations were high among clinicians concerning the therapeutic value of NOS inhibitors in septic shock patients. Unfortunately, a phase III randomized double blind placebo controlled study investigating the potentials of the NOS inhibitor, L-

NMMA, to reverse catecholamine resistant septic shock was prematurely terminated due to increased 28 day mortality in the treatment group, mainly of cardiovascular cause (58). This suggests that non-selective inhibition of the production of this important molecule hits to broadly, thus affecting fundamental regulation of the circulation. Principles of therapy targeting earlier steps in the cascade from pathogen to host NO production might be more successful.

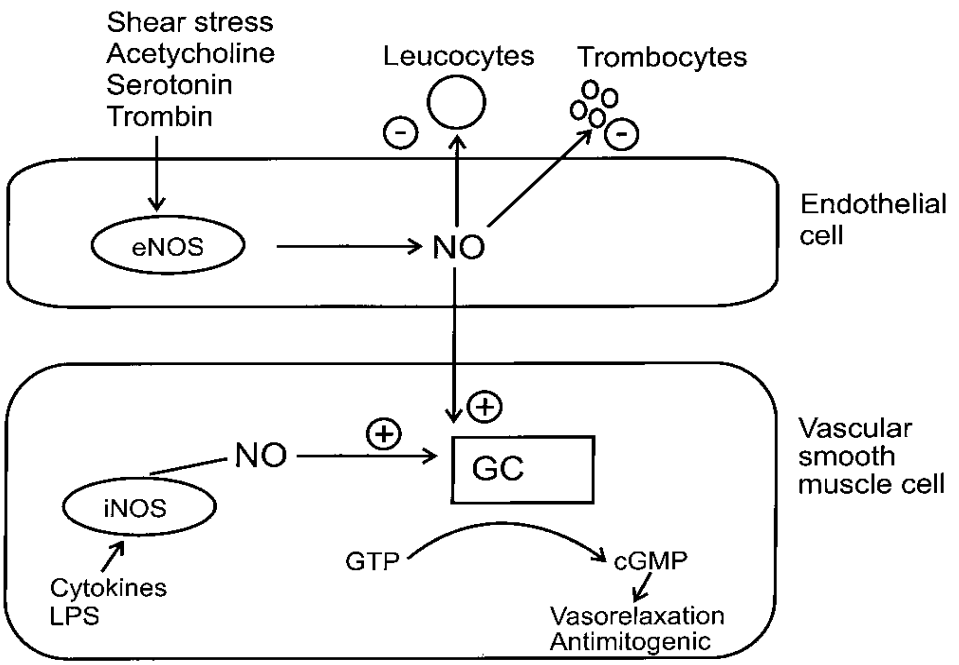

Fig 1. *Nitric oxide (NO) formation via inducible nitric oxide (iNOS). Endothelial shear or exogenous vasodilators release NO in the endothelium. NO rapidly diffuses into the vascular smooth muscle cell were it binds and activates soluble guanylate cyclase (GC) to generate cGMP. Key action of cGMP is to mediate uptake of Ca^{+2} into the sarcoplasmic reticulum, which promotes vasodilatation. NO is also able to inhibit platelet aggregation and leukocyte adhesion. Moreover, NO can be produced via upregulation of iNOS via cytokines or action of PAMPs on TLR. From Kirkeboen et Strand, Acta Anaesthesiologica Scandinavica 1999; 43: 275-288*

AIMS OF THE STUDIES

The present thesis aims at answering the following questions:

Can removal of N-terminal hydrophobic amino acids from LL-37 decrease its cytotoxicity as well as its inhibition by serum but preserve the antimicrobial actions and LPS-binding properties?

Is it possible, by the aid of computer simulation to identify shorter variants of LL-37 with further reduced toxicity but preserved antimicrobial and LPS-neutralizing effects?

Can soluble M1 protein from *Streptococcus pyogenes* induce TLR-mediated vascular nitric oxide production sufficient to cause hyporesponsiveness to the vasopressor phenylephrine?

Does GKE, a shorter variant of LL-37 identified in paper II have immunomodulatory effects like native LL-37?

MATERIALS AND METHODS

For details on the material and methods used in the present thesis, the reader is referred to the separate papers. All experiments were approved by the Regional Research Ethics Committee and the Animal Research Ethics Committee of Malmö/Lund.

Peptides used in paper I, II and IV

In paper I, LL-37 (aa 104 to 140 of the cathelicidin propeptide hCAP-18) was compared to two less hydrophobic fragments obtained by N-terminal truncation, named 106 (aa 106 to 140) and 110 (aa 110 to 140), a previously designed more hydrophobic variant, the 18-mer LLKKK and the bovine cathelicidin BMAP-27, concerning antimicrobial properties, inhibition by serum, lipopolysaccharide neutralization, chemotactic activity and toxicity against human erythrocytes and cultured vascular smooth muscle cells (Table 2).

Table 2. Amino acid sequences of the peptides used in paper I

Peptide	Amino acid sequence
LL-37	(N)-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-(C)
Fragment 106	(N)- GDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-(C)
Fragment 110	(N)- RKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-(C)
18-mer LLKKK	(N)- KLFKRIVKRILKFLRKLV -(C)
BMAP-27[a]	(N)-GRFKRFRKKFKKLFKKLSPVIPLLHL-am-(C)

[a] Note that the bovine cathelicidin BMAP-27 is amidated at the C-terminal end. The underlined amino acids of the 18-mer LLKKK have been changed compared to the sequence of the native peptide, LL-37.

In order to identify a region of LL-37 responsible for the antimicrobial activity of the peptide, we used computer simulation to search for amphipathic, helical regions, with a high predicted internal stability. A 21-amino-acid fragment

(GKE) was synthesized, the biological actions of which were compared to those of two equally long peptides derived from the N and C termini of LL-37 as well as native LL-37 (Table 3). Amphipathicity of idealized helices was investigated by generating helical wheel diagrams (Fig 2).

Table 3. Amino acid sequences of the peptides used in paper II

Peptide	Amino acid sequence	Helicalicity index
LL-37	(N)-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-(C)	5.08
LLG	(N)-LLGDFFRKSKEKIGKEFKRIV -(C)	0.97
GKE	(N)- GKEFKRIVQRIKDFLRNLVPR -(C)	5.16
FKR	(N)- FKRIVQRIKDFLRNLVPRTES-(C)	2.93

Antimicrobial assay

Antimicrobial activity was measured by using radial diffusion assay. Isolates of the following microbes were used in paper I and II; *Escherichia coli,*

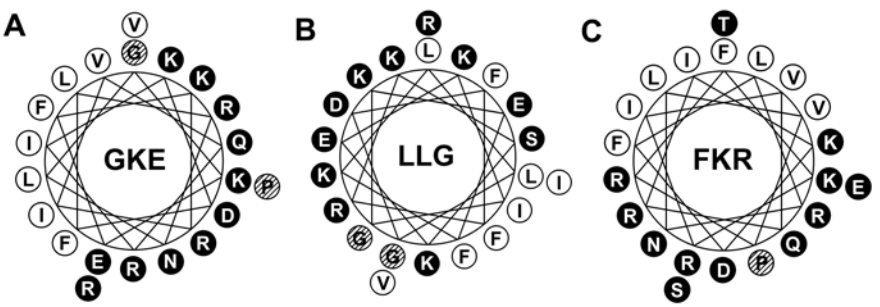

Fig 2 *Helical wheel diagrams of the peptides GKE (A), LLG (B), and FKR (C). Hydrophobic amino acids are represented by white circles, hydrophilic amino acids by black circles, and the indifferent amino acids, G and P, by hatched circles.*

Pseudomonas aeruginosa, *Staphylococcus aureus,* and *Candida albicans*. In paper II, an isolate of *Candida parapsilosis* was added. Microbes were mixed with agarose and allowed to solidify in a Petri dish. A series of 4 mm wells were punched into the gel after solidification and peptides in different concentrations were applied and allowed to diffuse into the gel. An overlay agar rich in medium to allow visible growth of the microbes was then poured over and the plates were incubated upside down for 18 h. Antibacterial activity was indicated by a clear zone corresponding to the lack of bacterial growth around the well. The diameter of the clear zone surrounding the wells was measured with a metric scale to the nearest 0.1 mm. In Paper I, minimal effective concentration (MEC) was defined as the lowest peptide concentration inhibiting visual microbial growth.

Assay of leakage from liposomes.

Unilamellar dioleoylphosphatidylcholine/cholesterol liposomes, around 100 nm in diameter, containing carboxyfluorescein (CF) were prepared. Intact liposomes contain CF at a high concentration at which the fluorescence is self-quenched and the recorded fluorescence intensity from liposomes with entrapped CF is therefore low. Upon leakage from the liposomes, released CF is dequenched and, hence, it fluoresces. The CF release was determined by monitoring the emitted fluorescence.

Effects of serum on antimicrobial activity.

Serum obtained from healthy human donors was mixed with peptide solutions to achieve a peptide concentration of 20 μM in 0, 40, or 99% serum. The peptide-serum mixtures were applied to the wells of a radial diffusion assay using *E. coli* as described above.

Cytotoxicity

Hemolysis

As an indicator for short term toxicity, the ability of the peptides to induce lysis of erythrocytes was tested. A suspension of erythrocytes from healthy human donors was incubated in the presence of peptides at different concentrations. Hemolysis was assessed by measuring the absorbance of the supernatant.

DNA fragmentation assay

DNA fragmentation is a part of the later stages of apoptosis. In order to investigate the cytotoxicity of the peptides after a longer exposure time we measured their ability to induce apoptosis in cultured human aortic vascular smooth muscle cells. Cultured human aortic vascular smooth muscle cells were incubated in the absence or presence of the peptides at different concentrations. DNA fragmentation was measured by binding of the DNA fragments to immobilized antihistone antibodies. The bound DNA fragments were then detected with anti-DNA antibodies.

Chemotaxis assay

Polymorphonuclear neutrophils (PMN) were isolated from healthy donor blood. Peptides at different concentrations were added to the lower wells of a microchemotaxis chamber. The suspension the PMN was added to the upper chamber, which was separated from the lower chamber by a polycarbonate membrane with pores so small that only activated neutrophils are able to pass through. After incubation for 30-60 minutes, the membrane was fixed, dried and stained. The number of transmigrated cells was counted in at least three fields with a light microscope and are expressed as number of cells per mm^2.

Measurement of nitric oxide production in blood vessels

Rats were killed under anestesthesia with isoflurane. The thoracic aorta was removed and cut into cylindrical segments. The segments were incubated for 24 h in the absence or presence of substances detailed in Table 4. As NO is

rapidly oxidized to nitrate and nitrite, NO release from the segments is reflected in the accumulation of nitrate and nitrite in the incubation medium. After incubation, the aorta segments were removed, briefly blotted on a paper cloth, and weighed. After centrifugation, the nitrite/nitrate concentration in the medium was determined using a colorimetric assay with Griess reagent.

Table 4. Substances used in experiments measuring vascular nitric oxide production.

Paper	Stimulant of NO production	Substance tested
I	LPS	LL-37 fragment 106 fragment 110 18-mer LLKKK BMAP-27
II	LPS	LL-37 GKE FKR LLG
III	LPS M1 protein	Dexamethasone
IV	IL-1β LTA M1 protein	GKE WRW4 WKY Kn62

Measurements of smooth muscle contraction of rat aorta

Rat aorta segments were incubated for 16 hours in the presence of the following combinations of compounds: a) control medium only, b) M1 protein or c) LPS. After incubation, the segments were placed on two L-shaped hooks

in 2-mL tissue baths containing Krebs-Ringer solution thermostatically maintained at 37 ºC and continuously aerated with 8% CO_2 in O_2. One of the hooks was connected to a force displacement transducer for isometric measurement of circular smooth muscle tension and the vessel tension was recorded. The α_1-adrenoreceptor agonist, phenylephrine, was added cumulatively. The resulting contraction was registered and concentration-response curves drawn. These experiments were performed in the absence or presence of the inhibitor of nitric oxide synthesis, L-N nitroarginine methyl ester (L-NAME).

Thin sectioning and transmission electron microscopy

Thin sections of aorta from wild type B6 BOM, knockout TLR2-/- and TLR4-/- mice, and human omental arteries and veins were prepared. M1 protein was labelled with 10-nm gold particles. Antibodies against TLR2 and TLR4 were detected with secondary antibodies labelled with 5-nm gold particles. The sections were incubated with labelled M1 protein and gold labelled antibodies and examined in an electron microscope. Thin sections from TLR2 -/- animals incubated with TLR2 antibodies and TLR4-/- sections with TLR4 antibodies were used as negative controls.

Measurements of smooth muscle contraction of mouse aorta

Wild type B6 BOM, TLR2 -/-, and TLR4 -/- mice were anesthetized to death with isoflurane. These experiments were performed essentially as previously described in rat aorta with minor modification.

RESULTS AND DISCUSSION

Paper I

LL-37 has an abundance of hydrophobic amino acids in its N-terminus. The removal of amino acids is named truncation. We chose to reduce the hydrophobicity of LL-37 in a stepwise fashion by removing the first two N-terminal leucines (resulting in fragment 106) and the first six amino acids, including the two leucines, one glycine, and two phenylalanines (resulting in fragment 110). All peptides tested were antimicrobial. Minimal effective concentrations are presented in Table 5. The presence of physiological salt concentrations did not affect the antimicrobial activities of the peptides to any major extent, with the exception that all the peptides in the concentrations tested lost their antimicrobial activities against *C. albicans* in the presence of 150 mM NaCl. Our data do not provide any explanation for this. It has previously been demonstrated that the helical structure of the LL-37 molecule is affected by the presence of anions such as Cl^- (39). It therefore seems reasonable to assume that the salt-dependent loss of activity against *C. albicans* is due to conformational changes of the peptide molecules. These experiments demonstrate that moderate N-terminal truncation does not reduce the antimicrobial action of LL-37 (Table 5).

The presence of serum markedly reduced the antimicrobial activity of LL-37, to a smaller extent fragment 110, but did not affect fragment 106 (Figure 3). Serum alone was weakly antibacterial. It has been demonstrated that the binding of LL-37 to the plasma protein apolipoprotein A-1 inhibits both its antimicrobial and cytotoxic effects (59). The fact that fragment 106 and fragment 110 were only marginally inhibited by serum indicates that the truncated amino acids are important for binding to plasma proteins, at least apolipoprotein A-1. Animicrobial acitivities of these fragments in serum must be regarded as beneficial if they are to be used as a systemic treatment for sepsis patients (42).

Table 5. Minimal effective concentration values obtained by radial diffusion assay for LL-37, fragments 106, 110 and 18-mer LLKKK as well as BMAP-27 against Gram-negative and -positive bacteria and *C. albicans*.[a]

	NaCl (mM)	Minimal effective concentration (μM)				
		LL-37	Fr 106	Fr 110	18-mer	BMAP-27
E. coli	0	0.26	0.40	0.33	0.33	0.14
	150	1.2	0.42	0.47	0.66	0.50
P. aeruginosa	0	0.18	0.26	0.18	0.29	0.20
	150	0.19	0.22	0.15	0.06	0.10
S. aureus	0	1.5	0.86	0.8	1.2	0.67
	150	4.6	2.6	2.4	0.39	0.45
C. albicans	0	10	5.1	2.4	2.2	0.36
	150	>40	>40	>40	>40	>40

[a]The values were determined at low (0 mM) or physiological concentration (150 mM) of NaCl. Each value is based on data from 3 independent experiments.

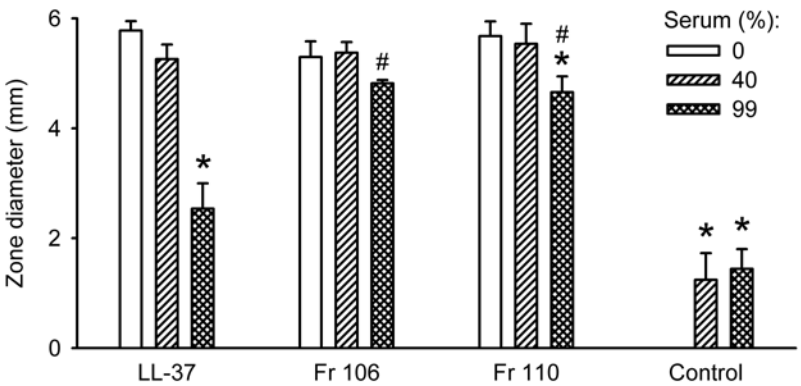

Fig 3. *Inhibition of antibacterial activity by serum, as assessed by a radial diffusion assay using E. coli.* *******statistically significant difference from experiments with the same peptide at 0 % serum (control) and # statistically significant difference from result in the presence of LL-37 at the corresponding serum concentration.*

All peptides tested inhibited nitrate/nitrite accumulation similarly (Fig 4). Polymyxin B was used as a positive control for LPS neutralization.

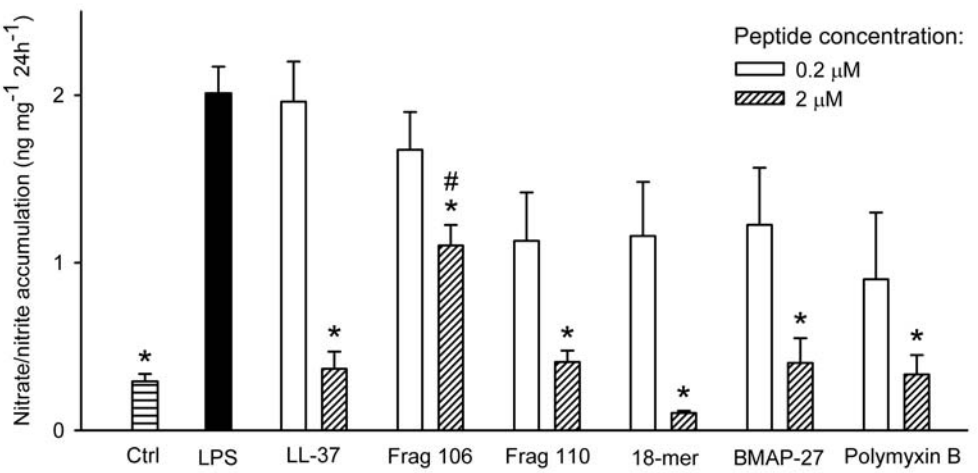

Fig 4. *Nitrate/nitrite accumulation in segments of rat aortas during 24 h of incubation, as measured with Griess reagent. * statitically significant difference from nitrate/nitrite accumulation in the presence of LPS and # statistically significant difference from effect of LL-37 at the corresponding concentration. LPS alone (filled bar) increased the amount of nitrate/nitrite production compared to control. Coincubation with LPS and peptides lowered the production of nitrite/nitrate induced by LPS.*

We conclude that N-terminal truncation of LL-37 does not affect LPS binding and neutralization to any major extent, which is consistent with previous findings that it is the middle portion of LL-37 that possesses the LPS neutralizing effects (60).

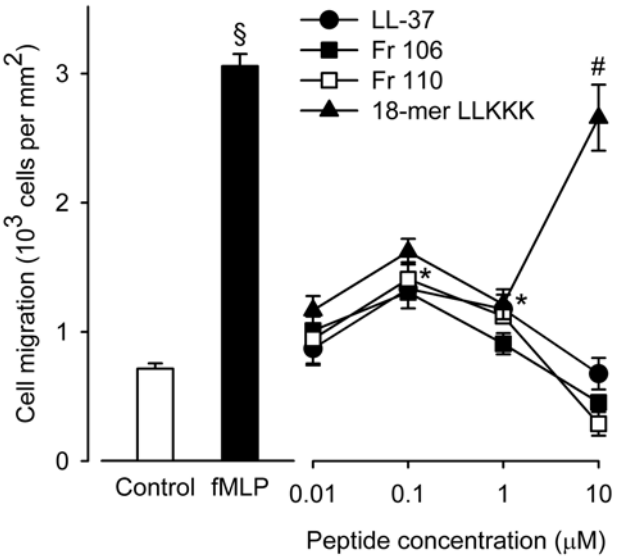

Fig 5. *Chemotaxis of human neutrophils in response to LL-37, fragments 106 and 110, 18-mer LLKKK, and the classical chemoattractant formyl peptide, fMLP, *statistically significant difference from control, # statistically significant difference from value for LL-37 at corresponding concentration.*

As expected, the positive control fMLP displayed a significant chemoattractant activity (61). The concentration-dependent chemoattractive action of the peptides was similar, forming a biphasic curve. The chemotactic function of neutrophil granulocytes seem to be decreased in septic patients, but the pathophysiological implications of this remain to be elucidated Infusion of the chemoattractant interleukin-8 or fMLP into rabbits abolishes the ability of neutrophil granulocytes to migrate into tissues, partly due to an inhibition of adhesion to the endothelium (62, 63). Moreover, pretreatment of neutrophil granulocytes with chemotactic mediators decreases their migration through endothelial monolayers in vitro (61).

These results indicate that the N-terminally truncated analogs of LL-37 are chemoattractant receptor agonists. Whether this is beneficial for the treatment of sepsis remains to be determined.

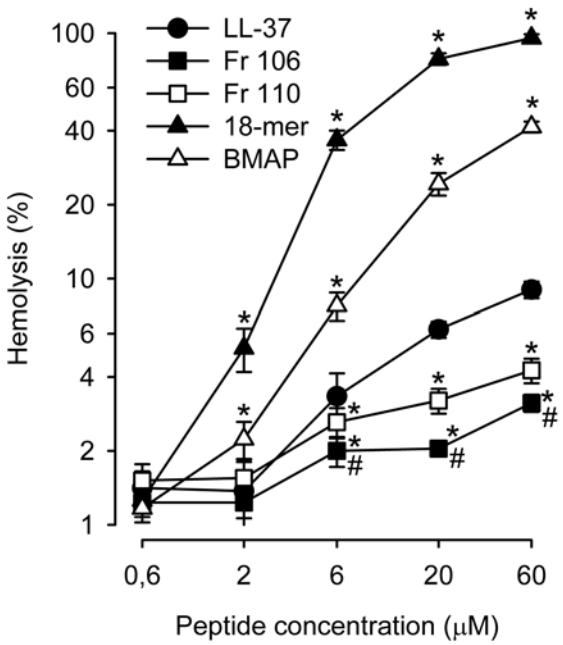

Fig 6. *Concentration-response curves of the lytic activity of the peptides towards human erythrocytes. * statistically significant difference from value for LL-37 at corresponding conc; # statistically significant difference from value for fragment 110 at corresponding concentration.*

All peptides induced a concentration-dependent hemolysis (Fig 6). 18-mer LLKKK and BMAP-27 showed severe cytotoxicity indicated by an even higher hemolysis than LL-37. On the other hand, our truncated variants Fr 106 and 110 caused less hemolysis than LL-37. The 18-mer LLKKK, has been demonstrated to be powerful at protecting mice from a lethal dose of endotoxin and was thus put forward as a candidate drug for the treatment of Gram-negative endotoxic shock (60). In our experiments, this peptide showed antimicrobial effects comparable to LL-37 and even greater LPS neutralization property but although it was reported not to be toxic to murine macrophages (60), we found it to induce far more haemolysis than LL-37 even at lower concentrations.

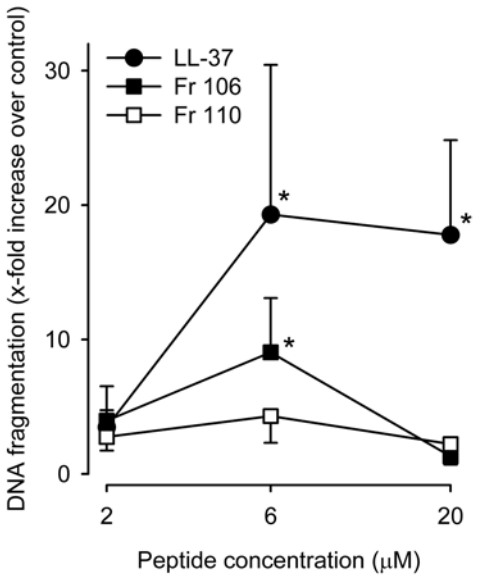

Fig 7. *DNA fragmentation in cultured human smooth muscle cells. * statistically significant difference from control in the absence of peptides.*

All peptides induced DNA fragmentation, but our two truncated variants to a much lesser degree than LL-37. 18-mer LLKKK and BMAP-27 were excluded from these experiments due to their high short term toxicity against erythrocytes.

Paper II

All peptides inhibited the growth of *E. coli* similarly at low salt concentrations. Our favourite GKE still showed antibacterial activities against *E. coli* in the presence of NaCl at 150 mM (physiological salt concentration) and, in fact, GKE inhibited the growth of *P. aeruginosa* even more efficiently than LL-37. In paper I, we showed that an enhanced activity against *S. aureus* can be achieved by removal of as few as 2 or 6 N-terminal amino acids from LL-37, see Table 1 and 3 (64). This correlates with the present finding that the mainly N-terminally truncated peptides, GKE and FKR displayed stronger antimicrobial activities against *S. aureus* than LL-37 and LLG. All peptides inhibited the growth of *C. albicans* and GKE was also found to be the most effective against *C. parapsilosis*.

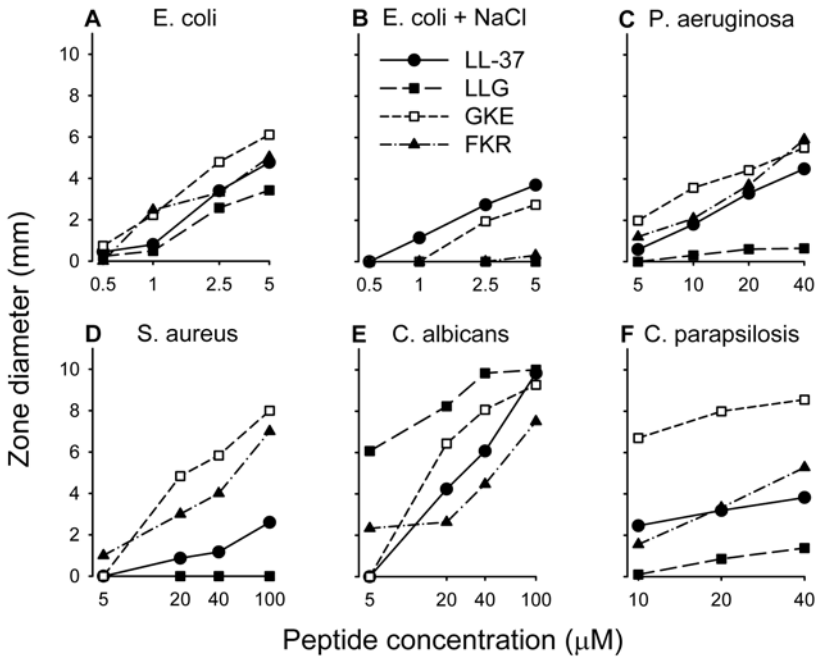

Fig 8. *Antimicrobial activity assessed by RDA*

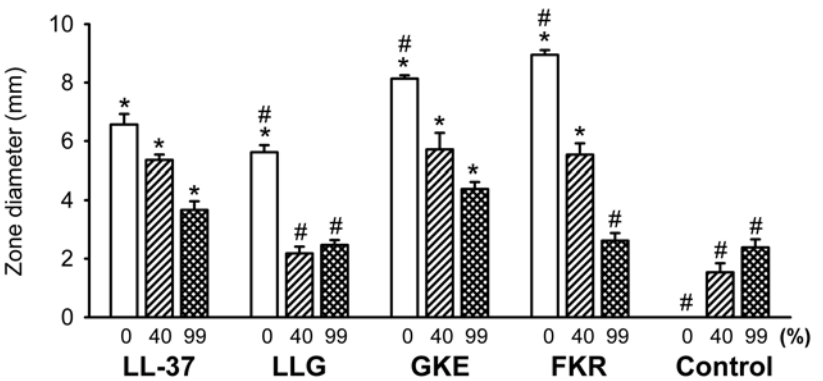

Fig 9. *Inhibition of antibacterial activity by serum assessed by radial diffusion assay using E. coli.* ******* *statistically significantly different from the value for serum alone at the same dilution, # statistically significantly different from the value for LL-37 in the presence of serum at the same dilution.*

GKE still retained antibacterial activity even in 99% serum (Fig 9). Further rendering it useful as a candidate for treating sepsis.

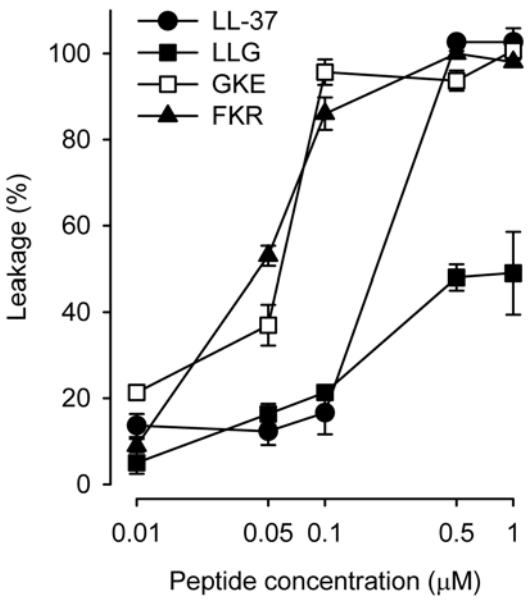

Fig 10. *Effects on liposomes*

Liposome experiments were performed in order to investigate if there was a connection between microbial killing and disruption of lipid membranes (Fig 10). As the leakage induced by the peptides found in the liposome experiments correlated with the antibacterial activity of the peptides, it seems reasonable to believe that the induction of microbial lysis is an important mechanism for the inhibition of bacterial growth by all these peptides. Interestingly, no such correlation was observed for the inhibition of the growth of the two *Candida* species suggesting that the mechanisms of the antifungal actions are different from their antibacterial actions.

GKE was as potent and effective in neutralizing LPS as intact LL-37 (Fig 11). Although conventional antibiotics may successfully kill pathogens involved in gram-negative sepsis, they cannot bind and neutralize LPS. In fact, bacteriolytic antibiotics, such as β-lactams, can even increase the amount of LPS (65). This means that despite the use of conventional antibiotics and support therapy, endotoxemia may still remain.

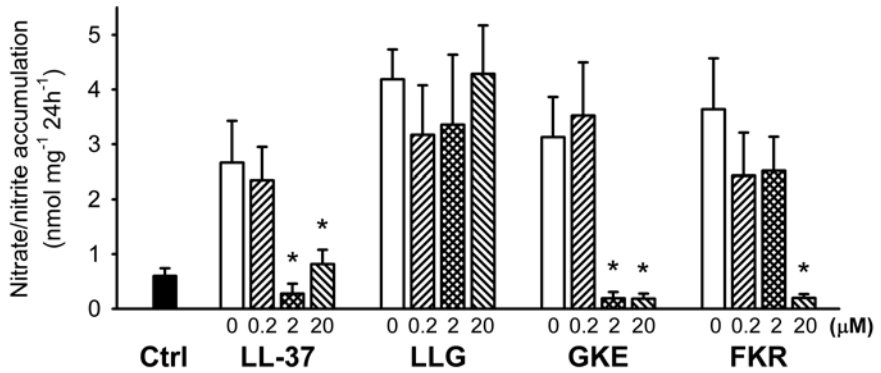

Fig 11. *Nitrate/nitrite accumulation.in segments of rat aortas during 24 h of incubation, as measured with Griess reagent. Open bars, LPS alone; * statistically. significant. difference from the value for LPS alone.*

LPS is one of the most powerful stimulants of the immune system (1). Thus, by binding and neutralizing LPS, it would be possible to avoid these mechanisms, which seriously contribute to the pathophysiology of sepsis.

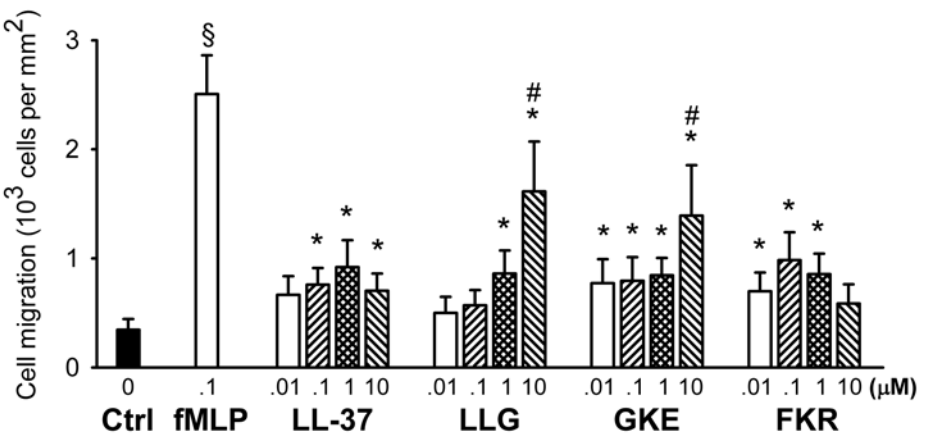

Fig 12. *Chemotaxis of human neutrophils in response to LL-37, LLG, GKE, FKR and the classical chemoattractant formyl peptide, fMLP. * statistically significant difference from control, # statistically significant difference from value for LL-37 at corresponding concentration.*

All the peptides tested possessed similar chemotactic activities against neutrophils (Fig 12). In the highest concentration tested, GKE showed a pronounced increase in its chemotactic activity. This could, at least partly, be due to a loss of selectivity at higher concentrations, resulting in an agonistic activity on other chemoattractant receptors than the receptor activated by LL-37, FPRL1.

The results of the cytotoxicity experiments in paper II, concerning both hemolysis and DNA fragmentation showed that our truncated variants were all significantly less toxic than the full-length LL-37. LL-37 has been found to prevent sepsis in LPS-exposed neonatal rats (66). However, our previous results show that even low doses of LL-37 can induce apoptosis in human cultured smooth muscle cells (6). Taken together this paper show that in silico analysis may be useful in the design of shorter peptides with a lower toxicity than the naturally occurring LL-37.

In this way we have identified GKE, a 21-amino-acid-long peptide constituting the midportion of LL-37, displaying antimicrobial and LPS-binding activities similar to those of LL-37 but which is less toxic. This peptide could serve as a template for the development of peptide antibiotics for the treatment of sepsis. LL-37 has recently been shown to have immunomodulatory effects on the host's defense against infections (67). We were thus interested to find out if the same applied to GKE. To this end we needed a system for vascular inflammation independent of LPS since the direct binding of GKE to LPS, neutralizing its actions could interfere with the interpretation of the immunomodulatory results. We therefore investigated the actions of M1 protein from *S. Pyogenes*.

Paper III

M1 protein induced NO formation in rat aorta comparable to LPS (Fig 13). These results indicate that M1 protein is a powerful inducer of vascular inflammation. NO is a vasodilator and causes hyporesponsiveness to vasocontrictors in vitro (68, 69). It is also well known that septic patients do not respond to vasoconstrictive drugs to the same extent as non septic patients, in part due to an increased basal NO-production (19, 70). We were therefore interested in investigating if M1 protein could also mediate the response of vasoconstrictors.

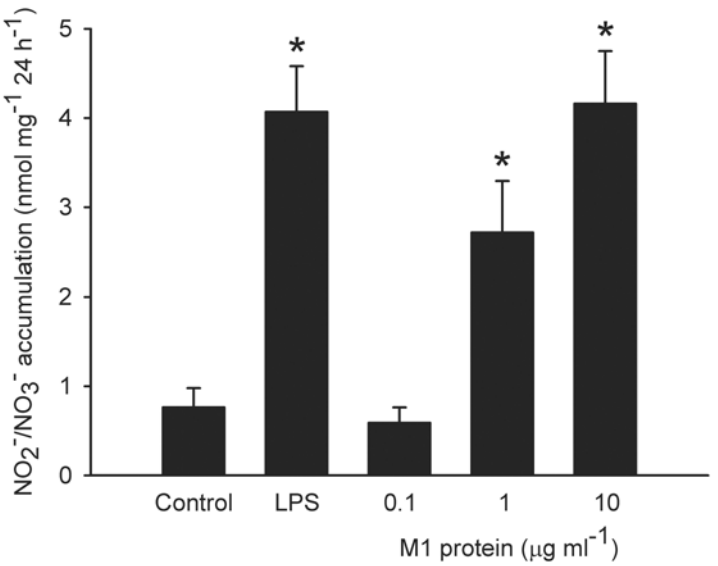

Fig 13. *Nitrite/nitrate accumulation in rat aorta incubated with LPS and M1 protein from S. pyogenes. * statistically significant difference from control.*

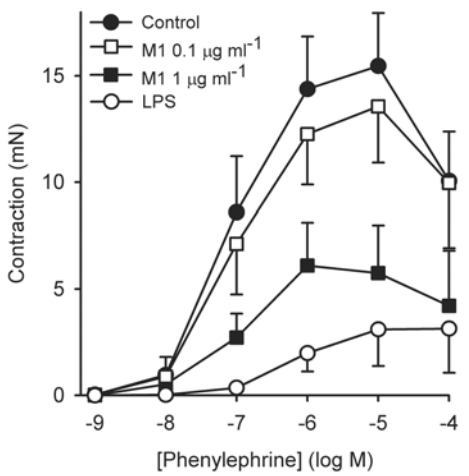

Fig 14. *Smooth muscle contraction in rat aorta in response to phenylephrine.*

These experiments demonstrated, that segments incubated in the presence of M1 protein or LPS showed a marked hyporesponsiveness towards the selective α_1-adrenoreceptor agonist, phenylephrine (PE), commonly used in clinical practise to treat hypotensive sepsis patients (Fig 14).

To investigate if this hyporesponsiveness of M1 towards PE was in fact due to NO and not any other mechanism, we added the nitric oxide synthase inhibitor, L-NAME (Fig 15).

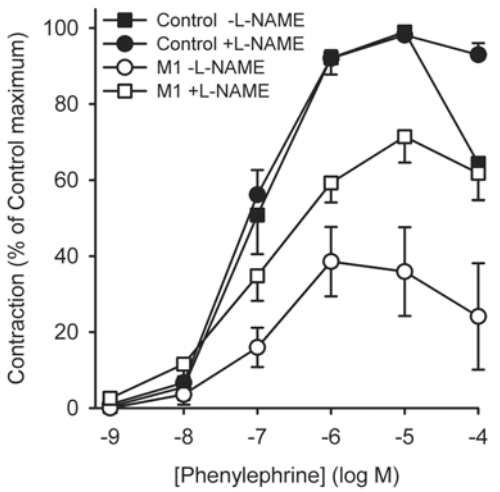

Fig 15 *Smooth muscle contraction in rat aorta in response to phenylephrine.in the presence and absence of L-NAME.*

The presence of L-NAME resulted in a greater response towards PE in the M1 protein pretreated segments (Fig 15). These findings suggest that the hyposensitivity to PE is indeed due to an M1 protein-induced increase in NO production. M1 protein-induced NO production was inhibited by dexamethasone to the same extent as the production induced by LPS (68). This inhibition of dexamethasone can at least partly be explained by decreased activation of NF-κβ and diminished iNOS mRNA transcription (71). The therapeutic value of corticosteroids in septic patients has been a long debate (72). The present results suggest that the modern clinical practise, which includes administrating low doses of these drugs to patients with vasopressor resistant circulatory failure due to sepsis, might be beneficial in both Gram-negative and Gram-positive sepsis (73).

To elucidate the receptors involved in the response to M1 protein we used immunohistochemistry at the electron microscope level (Fig 16).

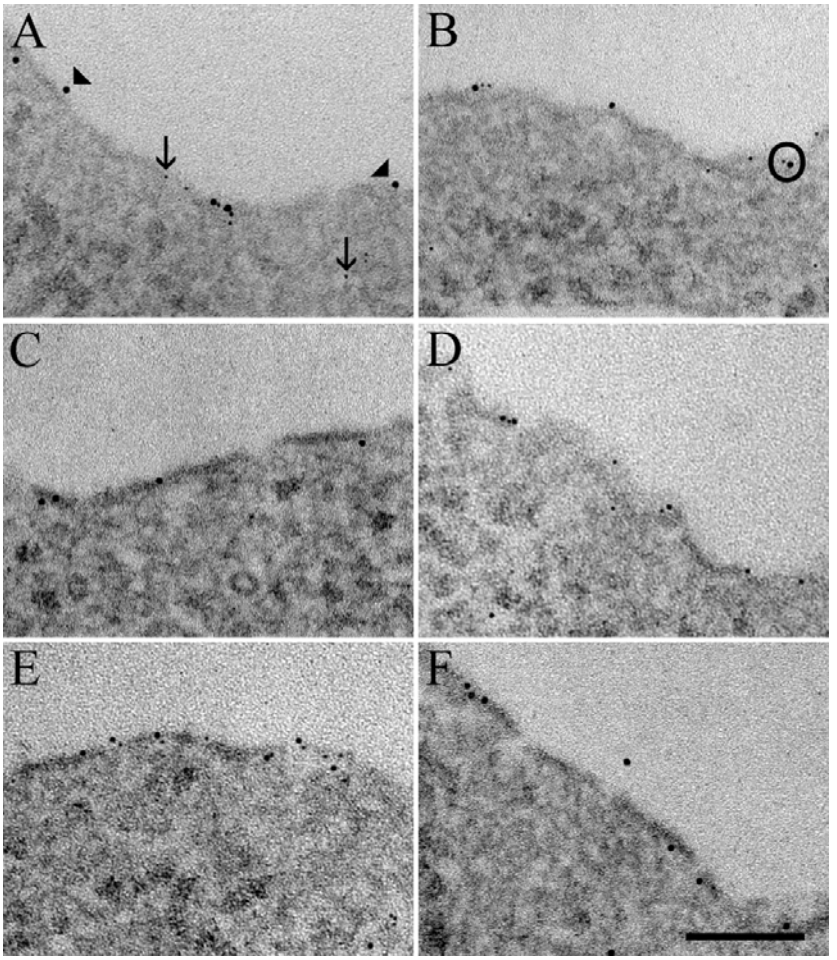

Fig. 16. *Ultra-thin sections of mouse aorta showing binding of M1 protein to TLR2 and TLR4 visualized with transmission electron microscopy. A and B are from a wild type, C and D from TLR2 -/- and E and F from TLR4 -/- mouse. In aorta from wild type mice, M1 protein (labelled with 10-nm gold particles, arrowheads) co-localized with antibodies (labelled with 5-nm gold particles, arrows) against TLR2 (A) and TLR4 (B) at the endothelial cell plasma membrane. TLR2 antibodies did not bind to aorta from TLR2 -/- mice (C) while M1 protein co-localized with TLR4 antibodies (D). Similarly, M1 protein co-localized with TLR2 antibodies (E) while TLR4 antibodies did not bind to aorta from TLR4 -/- mice (F). Bar, 100 nm. A pair of co-localized 10-nm and 5-nm gold particles, is shown in the circle.*

These experiments were performed in order to investigate if M1 protein, as several other Gram-positive bacterial products, could be a ligand for TLR2 in the blood vessel wall (74). The results from the experiments in mouse aorta revealed that M1 protein binds to both TLR2 and TLR4. However, co-localization in immunoelectron microscopy does not necessary mean that M1 functionally activates the receptors. In order to investigate if M1 functionally activated both TLR2 and TLR4 we performed smooth muscle contraction experiments (Fig 17).

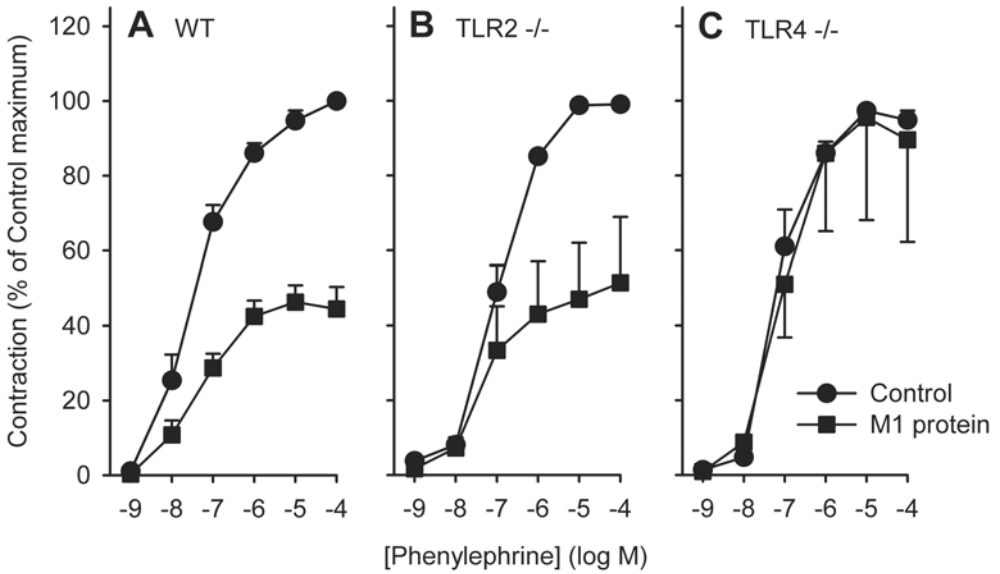

Fig 17. *Smooth muscle contraction in response to phenylephrine in aorta segments from wild type (A), TLR2 -/- (B) and TLR4 -/- (C) mice.*

Phenylephrine induced a concentration-dependent contraction in all the mouse aorta segments. The contractile response of the segments previously incubated with M1 protein (and LPS not shown) was weaker compared to control in the segments from WT and TLR2 -/- mice, while the contraction from TLR4 -/- mice was similar in all the segments, regardless of previous incubations.

The findings indicate that M1 protein is a functional ligand for TLR4 but not for TLR2 in mouse aorta, despite colocalization with both of these receptors in the immunogold experiments. To our knowledge, M1 is among the first Gram-positive bacterial products shown to induce a TLR4 activation. The fact that

M1 induced hyporesponsiveness towards PE in the TLR2 -/- but not in the TLR4 -/- mice rule out contamination of other bacterial compounds such as peptidoglycan and lipoteichoic acid well characterized agonists of TLR2.

In order to investigate if M1 did colocalize to TLR2 and or TLR4 receptors in humans we performed immunogold experiments with human arteries Fig 18.

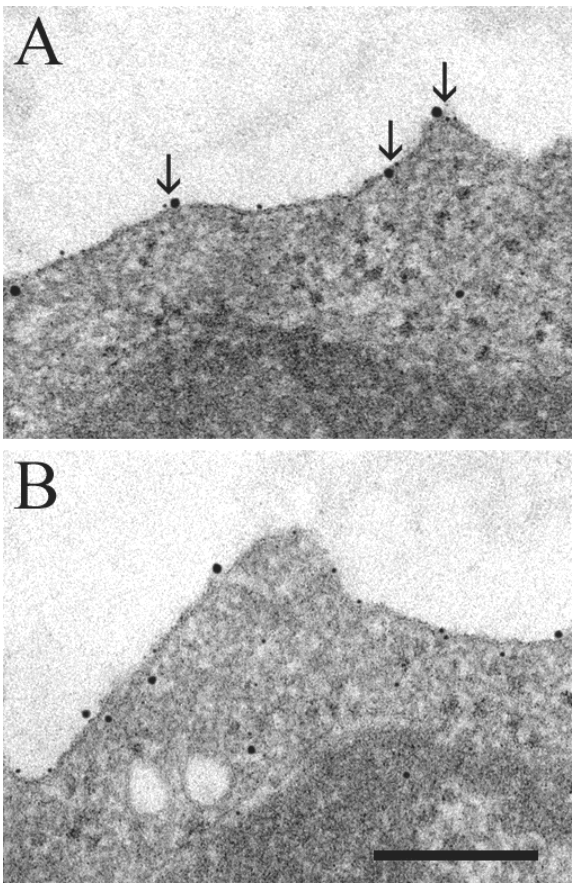

Fig 18. *Ultra-thin sections of human omental arteries showing colocalization of M1 protein to TLR2, indicated with an arrow (A) but not to TLR4 (B) visualized with transmission electron microscopy.*

Our results from the human immunogold experiments are in coherence with previous findings by Pahlman and collegues (51) indicating that M1 interacts with TLR2 on human peripheral blood monocytes and increases their

expression of various proinflammatory cytokines. The finding that M1 did not co-localize with the human TLR4 emphasises that results from animal experiments with PAMPs and their corresponding TLRs must be confirmed in humans. The gene sequence variation in TLR2 and TLR4 between human and mice could explain this difference (75). Another explanation for the discrepancy for M1 and TLR in mice and humans might be the fact that we used aortas from the mice but human omental arteries.

We conclude that M1 protein from *S. pyogenes* induces NO formation in rat aorta, sufficient to cause a significant hyporesponsiveness to PE. This could contribute to the severe hypotension we see in STSS patients (76). We also found that M1 protein from *S. pyogenes* can attach to both TLR2 and TLR4 but, at least in mouse aorta, only functionally activates the latter. M1 protein does, however, not interact with human vascular TLR4. Moreover, the results indicate that M1 protein could be used to induce an LPS-independent vascular NO production in a model to test immunomodulatory effects of antimicrobial peptides.

Paper IV

In order to investigate the effects of the LL-37 derived peptide GKE on vascular inflammation, we chose three previously known proinflammatory substances IL-1β, purified M1 protein from the outer wall of *S. pyogenes*, and LTA from *S. pyogenes*, all proposed to contribute to the hemodynamic and metabolic disturbances seen in septic shock and multiorgan failure (18-21).

Interleukin-1β induced a higher nitrite/nitrate production compared to control (Fig. 19). GKE at 0.2 μM inhibited the interleukin-1β induced nitrite/nitrate production but GKE at higher concentrations increased IL-1β induced NO-production.

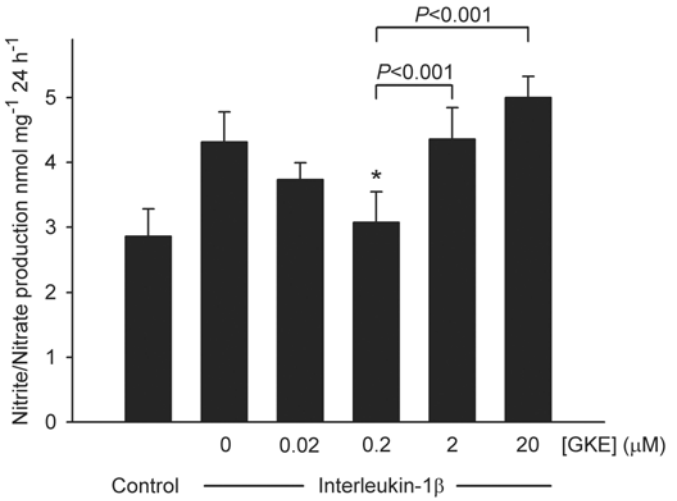

Fig 19. *Nitrite/nitrate accumulation in rat aorta segments after incubation with interleukin-1β, in the presence and absence of the LL-37 derived peptide GKE. * statistically significant. difference from value obtained by interleukin-1β in the absence of GKE.*

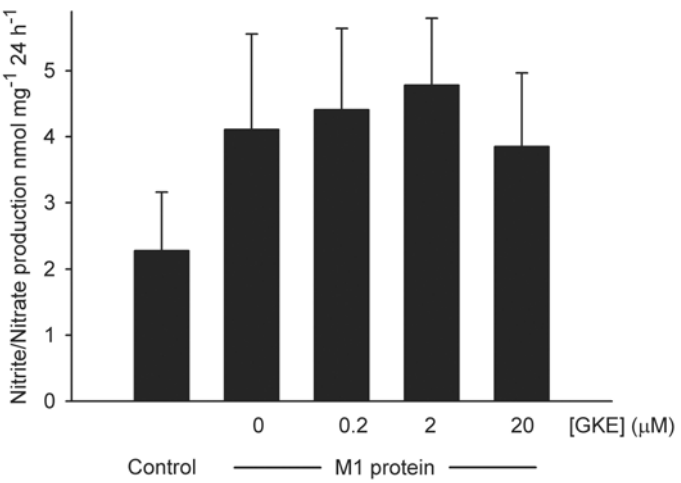

Fig 20. *Nitrite/nitrate accumulation in rat aorta segments after incubation with M1 protein from Streptococcus pyogenes, in the presence and absence of the LL-37 derived peptide, GKE.*

M1 protein induced a higher nitrite/nitrate production compared to control (Fig 20). GKE at the concentrations used did not affect the M1 protein induced nitrite/nitrate production

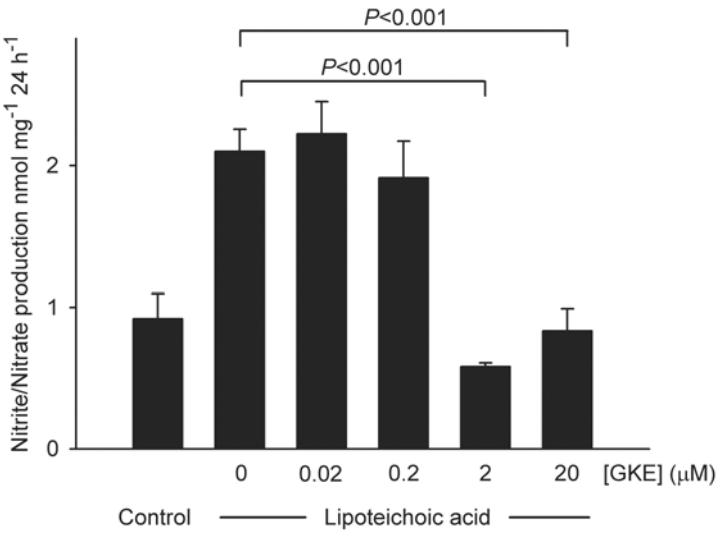

Fig 21. *Nitrite/nitrate accumulation in rat aorta segments after incubation with lipoteichoic acid from Streptococcus pyogenes, in the presence and absence of the LL-37 derived peptide GKE*

Lipoteichoic acid induced a higher nitrite/nitrate production compared to control (Fig 21). GKE at low concentrations did not affect the lipoteichoic acid induced nitrite/nitrate production while GKE at higher concentrations inhibited it.

IL-1β, M1 protein as well as LTA were thus found to induce NO formation in rat aorta, in coherence with the literature as well as unpublished observations (23, 24, Paper III).

The biphasic response of GKE regarding IL-1β, suggests multiple mechanisms of action by GKE. GKE did not, when incubated alone, have any effects on vascular NO formation. The fact that GKE did not affect M1 protein induced NO production but inhibited LTA-induced NO production, suggests that these two proinflammatory compounds from *S. pyogenes*, at least in rat aorta, induce vascular NO formation by different mechanisms. LTA is generally thought to

act primarily via toll-like receptor 2 (TLR2). There is evidence that M1 protein binds to TLR2 in human monocytes (21) and human omental arteries and veins (paper III, unpublished data). In experiments with mouse aorta, however, we found that M1 protein binds to both TLR2 and TLR4 receptor but only activates the latter (paper III, unpublished data). It is therefore plausible that M1-protein induced NO formation in rodents is mediated via TLR4. The different TLRs involved in cellular activation by LTA and M1 protein could be the reason for the differential effect of GKE.

We decided to further study the interplay between LTA and GKE due to the fact that attenuating LTA-induced secretion of vasodilators, such as NO, could be of great importance in combatting septic shock and multiorgan failure caused by Gram-positive bacteria (20). The experiments revealed that the anti-inflammatory effect brought about by GKE seems to involve neither the $P2X_7$ receptor nor FPRL1 receptor as their antagonists Kn62 and WRW4, respectively, did not inhibit the attenuating action of GKE on LTA-induced NO production (data not shown).

In conclusion, the candidate previously suggested for peptide based treatment of sepsis, GKE, seems to possess complex immunomodulatory effects.

CONCLUSIONS

Removal of hydrophobic amino acids from LL-37 decreases its cytotoxicity as well as its inhibition of serum but preserves the antimicrobial actions and LPS-binding properties.

By computer simulation we have identified GKE, a 21amino acids long peptide constituting the midportion of LL-37. GKE displayes antimicrobial and LPS-binding activities similar to those of LL-37 and is less cytotoxic.

Soluble M1 from *Streptococcus pyogenes* induces NO formation sufficient to cause hyporesponsiveness to phenylephrine, at least in mouse aorta mediated via TLR4.

GKE, a shorter version of LL-37, developed as described in Paper II, possesses complex immunomodulatory effects.

SUMMARY IN SWEDISH

Antimikrobiella peptider är kroppsegna antibiotika som frisätts vid infektioner och inflammationer. LL-37 är ett spiralformat sådant som består av 37 aminosyror. Det har inte endast ett brett antimikrobiellt spektrum utan binder och neutraliserar även endotoxiner som finns i Gram-negativa bakteriers vägg. Endotoxiner har stor betydelse för utvecklingen av den svikt av livsviktiga organ som kan bli följden av svåra intensivvårdskrävande infektioner såsom blodförgiftning (sepsis). Endotoxiner kan nämligen stimulera kärlen att bilda kvävemonoxid som i sin tur leder till att kärlen vidgar sig vilket kan bidra till det blodtrycksfall man ser vid allvarlig blodförgiftning.

LL-37 skulle alltså kunna användas som ett läkemedel vid allvarlig blodförgiftning. Tyvärr är LL-37, som det förekommer i kroppen, giftigt mot våra celler och därmed inte användbart, trots sina stora fördelar.

Delarbete I. Vissa fettlösliga delar av LL-37 skulle kunna ligga bakom giftigheten. Vi lät därför syntetisera två varianter av LL-37 som inte var lika fettlösliga. Dessa LL-37-varianter visade sig vara minst lika aktiva mot mikroorganismer och kunde även binda endotoxin och neutralisera dess stimulerande effekt på kväveoxidbildningen som LL-37, men var mindre giftiga mot mänskliga celler som mätt med två olika metoder.

Delarbete II. Vi använde datorprogram för att identifiera spiralformade delar av LL-37 som man tidigare har kopplat till dess förmåga att döda microorganismer. Vår hypotes var att man skulle kunna framställa kortare och mindre giftiga varianter av LL-37 förutsatt att spiralstrukturen är bevarad. Vi hittade ett lovande 21 aminosyror långt fragment, GKE, som visade sig ha minst lika stor antibiotisk effekt mot bakerier och svamp som är ofta orsakar sepsis som LL-37. GKE kunde även binda och neutralisera endotoxiner. GKE visade sig även mindre giftig mot mänskliga celler. Vi hoppas därför att GKE skulle kunna vara användbar som en ny peptidbaserad behandling av sepsis.

Delarbete III. Septisk chock och multiorgansvikt är en huvudorsak till mortalitet på intensivvårdsavdelningen. Infektion med den Gram-positiva bakterien *Streptococcus pyogenes* är en vanlig utlösande faktor. Streptokocker som uttrycker M-proteiner i sin cellvägg bedöms vara extra farliga. M-protein är en familj av olika faktorer som bidrar till bakteriernas förmåga att infektera. Man har nyligen visat att M1-proteiner (som oftast bidrar till allvarliga invasiva streptocockinfektioner) kan aktivera vita blodkroppar och därmed

påverka kroppens immunförsvar att bekämpa infektioner. Vi ville se om M1 protein kunde liksom endotoxin från Gram-negativa bakterier stimulera kvävemonoxid-bildning i blodkärl och därmed bidra till det besvärliga blodtrycksfallet som man ser hos dessa patienter. Det visade sig att M1-protein gav en kvävemonoxid-bildning som var så pass stor att den försämrade kärlens förmåga att dra ihop sig vid tilförsel av ett känt blodtrycksförhöjande läkemedel, fenylefrin. Det visade sig även att vissa receptore, vilka benämns Toll-like receptorer, bidrar till denna kvävemonoxid-bildning.

Delarbete IV. Naturligt förekommande LL-37 har visat sig kunna påverka immunförsvaret på olika sätt, och vi ville se om GKE, som vi identifierat i delarbete II också hade en sådan förmåga. Det visade sig att GKE på ett komplicerat sätt kan påverka immunförsvaret.

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