Anti-endotoxin properties of cationic host defence peptides and proteins

Dawn M.E. Bowdish, Robert E.W. Hancock

Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, British Columbia, Canada

The innate immune system of mammals contains a series of peptides with overall positive charge and an amphipathic structure which have a variety of important properties in host defences. Although these are often termed cationic antimicrobial peptides, they have numerous roles in innate defences in all complex species of life and thus we prefer to refer to them as host defence peptides. These roles include: (i) an ability to kill micro-organisms directly, ranging from bacteria to viruses, fungi, parasites and helminths; (ii) an adjuvant activity in the adaptive response; and (iii) a multiplicity of roles in modulating innate immunity, including an apparent ability to stimulate protective innate immunity while suppressing harmful inflammatory/septic responses. This latter property may be one of the more important activities of these peptides *in vivo*. Innate immunity is thought to be triggered by the interaction of conserved bacterial components with particular receptors including Toll-like receptors (TLRs) on host cells. However, the initiation of the innate immune response through this route may trigger a pro-inflammatory cascade that is the principle cause of harmful conditions such as sepsis. Since we are exposed to potentially dangerous pathogens on a daily basis, the host response must contain certain checks and balances. We propose that host defence peptides have a role in feed-back modulation of inflammation under normal (low-pathogen exposure) conditions. This review surveys the available information regarding the anti-endotoxic/anti-inflammatory properties of host defence peptides, and will address whether this potential might be exploited for therapeutic benefit in sepsis.

Keywords: Host defence peptides, sepsis, immunomodulator, endotoxin

Sepsis and septic shock

In the industrialised world, where a high percentage of infections can be managed with antibiotics, there has been a strong decline in deaths due to infectious diseases. Despite major advances in the treatment of infectious diseases in the past century, there has been very little improvement in the survival rate of patients suffering from severe sepsis and, under standard supportive care, mortality remains unacceptably high at 28–50%.[1,2] An estimated 751,000 individuals in North America annually suffer from a syndrome termed sepsis[3] causing 215,000 deaths. The costs of treating patients with severe sepsis in US hospitals are about $17 billion annually, making this one of the most serious maladies in Western society.

Sepsis has been defined as ‘a severe illness caused by overwhelming infection of the bloodstream by toxin-producing bacteria’; however, it has become more common to define

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Correspondence to: Bob Hancock, Centre for Microbial Diseases and Immunity Research, University of British Columbia, Room 225, 2259 Lower Mall Research Station, Vancouver, British Columbia V6T 1Z4, Canada
Tel: +1 604 822 2682; Fax: +1 604 827 5566;
E-mail: bob@cmdr.ubc.ca

Abbreviations: TLR, Toll-like receptor; LPS, lipopolysaccharide; LTA, lipoteichoic acid; BPI, bactericidal/permeability increasing protein; $rBPI_{21}$, recombinant bactericidal/permeability increasing protein 21-kDa fragment; LBP, lipopolysaccharide binding protein

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sepsis based on the presence of documented or suspected infection accompanied by symptoms such as fever, hyperventilation, chills, shaking, skin rash, rapid heart beat, confusion or delirium.[4] Generally, sepsis is initiated when an infection breaches the site of initial infection, resulting in a systemic response to pathogens, termed systemic inflammatory response syndrome (SIRS). SIRS involves a disruption of homeostatic controls leading to an uncontrolled cascade of inflammation and coagulation, and impaired fibrinolysis. This may result in septic shock, which is a complex syndrome that occurs as a result of disturbed microcirculatory function leading to global tissue hypoxia that is a major cause of tissue damage. This can ultimately lead to multi-organ failure and a high frequency of death. The sequence of events leading to septic shock is not completely understood and is not expected to be completely universal as both the underlying diseases and the root causes can vary.[5] There are many root causes of septic shock including burns and trauma, acute pancreatitis or infection with certain viruses, but by far the most common cause is infection by Gram-negative and Gram-positive bacteria. Antibiotic treatment of these infections is not necessarily sufficient and it is common for individuals who have developed systemic infections, and then have been treated with antibiotics, to develop profound problems associated with sepsis and even to succumb to this syndrome.[6,7]

There are many points in the inflammatory cascade leading to organ dysfunction and failure that show potential as therapeutic targets. Despite many studies in animals and several clinical trials, there is only one product which has received FDA new drug approval, namely Xigris which is an active recombinant form of activated protein C that is proposed to intervene in and modulate the sepsis cascade due to its anticoagulant functions. However, this treatment has shown only modest success, reducing the risk of death due to sepsis by only 6%. Other proposed treatments including inhibitors of pro-inflammatory cytokine production, antibodies against endotoxins or key cytokines, inhibitors of other soluble mediators,[8] cytokines such as G-CSF,[9] extracorporeal endotoxin removal[10] and LPS agonists[11] have met with mixed success. One of the difficulties in translating the results of animal models to clinical trials is that there are a wide variety of ways to induce a septic condition in mice, none of which accurately reflects the evolution of disease from an initial site of infection to a full-blown systemic response in humans and/or accounts accurately for the immunological (*e.g.* immunosuppression) or genetic variability that predisposes the host to the development of sepsis. Indeed, septic shock manifests so differently between adults and paediatric patients that different criteria must be used for paediatric patients.[4] Despite difficulties in diagnosing and characterising septic shock and the failure to produce an appropriate animal model to predict the effectiveness of treatments, there have been a number of potential therapeutic agents identified. One class of these agents are the cationic host defence peptides, some of which possess both antimicrobial and anti-septic properties

Host defence peptides as anti-sepsis agents

Host defence peptides are small, positively charged peptides which are an evolutionarily conserved component of the innate immune response. Individual peptides are found in high concentrations in the granules of neutrophils and some can be produced by epithelial and other cell types upon stimulation with bacterial components or inflammatory mediators.[12,13] Originally, these peptides were believed to function simply as natural antibiotic compounds in the antibacterial defences of neutrophils; however, it has become apparent that their antimicrobial activity extends to viruses and eukaryotic microbes, and they appear to enhance host defences by interacting with neutrophils,[14] monocytes,[15] macrophages,[16] dendritic cells,[17] T cells,[14] and epithelial cells.[18,19] Indeed, it has been proposed that host defence peptides are evolutionarily related to chemokines as a common feature of mammalian host defence peptides is their ability to stimulate chemotaxis of a number of different cell types and conversely many chemokines have been demonstrated to have antimicrobial properties.[20,21] Although the mechanisms by which these peptides kill bacteria are not fully elucidated, many mammalian antimicrobial peptides kill bacterial cells in a non-lytic manner.[22] This is an especially valuable asset considering that the use of antibiotics which lyse bacteria, and consequently release immunostimulatory bacterial components has been linked to the development or enhancement of septic shock.[6,7] Early experiments also determined that a number of host defence peptides from various sources bound to diverse chemotypes of LPS and reduced LPS-induced release of pro-inflammatory cytokines (*e.g.* TNF-α, IL-1, IL-6) and nitric oxide from monocytes or macrophages[23–26] although these two observations are not obligately linked.[27] Nevertheless, it has been clearly demonstrated that various host defence peptides protected mice from LPS-induced lethality.[26, 29–32] As the excessive production of pro-inflammatory cytokines precedes the development of full-blown septic shock, the ability of these peptides to reduce pro-inflammatory cytokine production induced by a variety of pathogen molecules, including LTA, CpG and LPS, indicates that they might function as broad-spectrum anti-sepsis agents.[33,34]

Bactericidal/permeability increasing protein (BPI)

In vitro observations

BPI was originally discovered as a cationic protein, from the granules of neutrophils, that enhanced bacterial permeability and was antimicrobial towards Gram-negative

but not Gram-positive organisms.[35,36] BPI from both rabbit and human sources kill Gram-negative bacteria in a non-lytic manner[37] and neutralised the pro-inflammatory properties of LPS.[38,39] Thus BPI is believed to be an important component of innate host defences and a major component of non-oxidative killing by neutrophils.

The structure of BPI has been resolved and reveals a boomerang-shaped structure with two hydrophobic pockets opening on its concave structure, which in the crystal structure were filled with lipid molecules making these the candidate LPS binding pockets.[40] It is a large protein (60 kDa) and thus was not considered to be a likely target for drug development until it was determined that a recombinant 25-kDa N-terminal fragment of BPI had both antimicrobial and LPS neutralising activities.[41] BPI has a very high affinity for binding to LPS with a K_d of 2–5 nM.[42] Although BPI is larger than most host defence peptides, it is related in that there are a variety of synthetic peptide derivatives of BPI that have high binding affinities for both lipid A and LPS and neutralise activity *in vitro* and *in vivo*.[43]

It has been proposed that the majority of the observed protective effects are due to the ability of BPI (and its derivatives) to bind and neutralise LPS. Thus, BPI has been shown to reduce LPS-induced, but not IL-1β-induced, NO synthesis.[44] The ability of BPI to sequester LPS is predicted to prevent a wide range of the physiological effects of Gram-negative septic shock. For example, it is believed that preventing LPS-induced changes in endothelial function are key to the prevention of many of the harmful consequences of loss of vascular tone. The endothelial cells which line the blood vessels display a variety of adhesion molecules for circulating white blood cells. They are extremely responsive to soluble mediators such as cytokines, leukotrienes and nitric oxide. In sepsis, these endothelial cells lose their anticoagulant function and display an increased number of adhesion molecules, which affects diapedesis and thus the recruitment of blood cells to the site of infection. Endothelial cells stimulated by LPS express E-selectin, which is a ligand for leukocytes. Increased levels of E-selectin are believed to contribute to the arrest and diapedesis of leukocytes as reviewed previously.[45,46] $rBPI_{21}$ (a recombinant 21-kDa N-terminal LPS-binding portion of BPI) reduces LPS-induced E-selectin when added concurrently with LPS or as much as 6 h afterwards.[47] Since the delayed addition of $rBPI_{21}$ also reduced LPS-mediated activation of the key pro-inflammatory transcription factor NF-κB, this implies that LPS must be continually present to induce pro-inflammatory signalling maximally and stimulate endothelial changes.[47] It has been demonstrated that BPI and its derivatives reduce LPS-induced TNF-α production *in vivo* from primary mononuclear cells, reduce NO and pro-inflammatory cytokine production from monocytes/macrophages, as well as kill bacteria and reduce TNF-α in whole human blood.[48–51]

In vivo applications – animal studies

$rBPI_{21}$ has shown tremendous potential in animal trials, increasing survival in various mouse and rat models of sepsis as well as decreasing serum endotoxin and pro-inflammatory cytokine levels.[52–54] Other animal models have demonstrated the protective effects of BPI against acute endotoxin-induced lung injury,[55] and cerebrospinal fluid inflammation.[56] However, trials in primates have been more equivocal and although an initial trial in primates suggested that the full-length rBPI and a derivative could reduce circulating LPS and pro-inflammatory cytokine levels,[32] a subsequent trial did not demonstrate any increase in survival.[57]

Clinical trials

An initial phase I/II trial of $rBPI_{21}$ performed on paediatric patients suffering from meningococcal sepsis indicated that $rBPI_{21}$ could be given to paediatric patients, and the clinical outcome for these patients was better than expected when compared to historical controls.[58] However, in phase III clinical trials using $rBPI_{21}$ against paediatric meningococcal sepsis, no reduction in mortality was observed and was ascribed to the majority of deaths occurring in the interval between identification of patients and administration of $rBPI_{21}$. Consequently, there is no evidence that treatment with $rBPI_{21}$ can reduce mortality. However, patients receiving $rBPI_{21}$ demonstrated a moderate trend towards improved outcome and required fewer amputations.[59]

Cathelicidins

In vitro observations

The cathelicidins are host defence peptides found at high concentrations in the granules of neutrophils. Homologues in mice, cows, sheep, pigs, humans, and rabbits have been discovered.[21] These peptides are characterised by their evolutionarily-conserved N-terminal cathelin domain/pro-piece which is cleaved from the antimicrobial/immunomodulatory C-terminus, apparently upon degranulation from neutrophils. Cathelicidins from a number of species have been found to have LPS-binding and neutralising properties. Humans have one cathelicidin, named hCAP-18 in its unprocessed form, that can be processed to several peptides including the 37-amino acid peptide called LL-37.[60] Initial studies focused on the unprocessed form of LL-37, hCAP-18;[26] however, it was later found that the LPS binding properties of the peptide were contained within the processed peptide LL-37.[61] It has been proposed that the anti-endotoxic

properties of this peptides are due, in part, to an inhibition of the binding of LPS to CD14[62] and LBP,[27] although it is worth mentioning that the binding affinity of LL-37 to LPS is at least 100-fold less than BPI, and less than both lipopolysaccharide binding protein (LBP) and the cell surface molecule/receptor component CD14. Unlike BPI, which demonstrates antimicrobial activity under conditions mimicking those found *in vivo*, we have proposed that it is unlikely that the antimicrobial properties of LL-37 occur under physiological concentrations, as no antibiotic activity could be demonstrated in tissue culture medium.[63] The ability of cathelicidins to bind to LPS and block LPS-induced pro-inflammatory cytokine production seems to be conserved in all identified homologues to date, including those found in sheep,[64] multiple cathelicidins from cows,[65,66] and humans.[24] LL-37 has been shown to block a number of LPS-induced inflammatory responses including contractility and NO release in aortic rings,[67] infiltration of leukocytes and chemokine production,[30] and pro-inflammatory cytokine production in a macrophage cell line or in whole human blood.[29] It is possible that peptides like LL-37 at physiological concentrations may provide homeostatic buffering of the effects of low concentrations of LPS or other pathogen components.

In vivo applications – animal studies

In animal models, cathelicidins have been demonstrated to reduce LPS-induced TNF-α production,[29,30] suppress leukocyte infiltration in a model of endotoxin-induced uveitis,[30] and prevent lethality in animal models of sepsis in which the animals are injected with high concentrations of bacteria or LPS.[29,68] SMAP-29, an ovine peptide, has been shown to be protective in cecal ligation and puncture models of polymicrobial sepsis and to reduce pro-inflammatory cytokine production, and circulating levels of endotoxin.[64]

The majority of studies on the ability of host defence peptides to block experimental septic shock focus on Gram-negative aetiology. There is substantial *in vitro* evidence suggesting that these peptides can also block responses to Gram-positive organisms or Gram-positive cell wall components.[33] It is unfortunate that Gram-positive models of septic shock are understudied as the incidence of Gram positive induced shock has been sharply increasing.[2] As it is often difficult to assess accurately the causative agent of septic shock, the ideal therapeutic agent would demonstrate efficacy towards both Gram-positive and Gram-negative organisms. The bovine cathelicidin, BMAP-28 has been demonstrated to reduce LTA-induced TNF-α and NO production in a mouse macrophage cell line and to reduce lethality and blood counts in *Staphylococcus aureus* induced sepsis.[31] Although it is not as efficient at reducing blood counts of bacteria as conventional antibiotics, it demonstrated similar efficacy in preventing death when added simultaneously or 360 min after administration of the bacteria.[31]

Insect-derived peptides (CEMA, mellitin)

A prominent class of insect peptides is α-helical in structure and can be isolated from broad classes of insects. Two peptides that showed early therapeutic promise were cecropins (from silk moths) and melittin (from honey bees). Hybrids of the two that retained excellent broad-spectrum antimicrobial activity but were less toxic were produced, including the molecule CEME and its derivative CEMA (also called MBI-27 and MBI-28, respectively). These derivatives reduced TNF-α production from a mouse macrophage cell line stimulated with LPS and were protective in neutropenic mice challenged with *Pseudomonas aeruginosa* or in galactosamine-sensitive mice challenged with *Escherichia coli* LPS.[69] In addition, CEMA reduced the amount of circulating TNF-α in the blood of mice challenged with *E. coli* LPS.[69] However, to date, these have not been considered for clinical trials.

Mechanism of action

There is some *in vitro* evidence that the inhibition of pro-inflammatory cytokine production induced by bacterial components correlates with binding and neutralisation of these components by host defence peptides.[27] Host defence peptides and synthetic derivatives have been demonstrated to bind to the anionic sugars, phosphate groups and lipid A core of LPS, and this LPS binding is an important requirement for bacterial killing.[70,71] Indeed, it has been proposed that a conserved LPS binding motif exists which consists of both basic and hydrophobic amino acids and accounts for the ability of natural and synthetic peptides to bind and neutralise LPS.[72] LPS binding of antimicrobial peptides was indeed proposed to mask its biological effects,[69,73,74] in that high-affinity binding induces changes in the endotoxic LPS aggregate structure and a neutralisation of the negative charges of LPS. Nevertheless, some doubt exists as to whether LPS binding is the only mechanism involved in endotoxin neutralisation, as most peptides have LPS binding affinities that are orders of magnitude lower than that of BPI and yet are as effective, or in some cases more effective, at preventing mortality in animal models of septic shock. It has not been conclusively demonstrated that the concentrations of peptides used in these studies are sufficient to sequester all of the LPS at particular body sites in animal model protection studies. Similarly, although LPS binding affinity correlates with

Table 1. Animal models for testing the anti-endotoxic effects of host defence peptides

Animal model	Corollary in human disease	Demonstrated protective effect by host defence peptide	References
Cecal ligation and puncture	Polymicrobial bacteremia	SMAP-27, indolicidin, protegrin derivative	64,75,76
Injection with bacteria/bacterial components and simultaneous treatment with peptides	Rapid response to initial stages of infection?	BPI and derivatives, LL-37, BMAP-28, indolicidin, protegrin derivative	29–32,75,76
Injection with bacteria/bacterial components and delayed treatment with peptides	Bacteremia	SMAP-27	64
Burn	Burn triggered contractile dysfunction	rBPI	77
Neonatal animals injected with bacteria/bacterial components	Paediatric bacteremia	LL-37	68

an ability to block TNF-α production from a macrophage cell line there are certain peptides, such as a derivative of bovine bactenecin, that has a relatively high binding affinity for LPS but is virtually unable to block LPS-induced cytokine production.[27,66] There is emerging evidence suggesting that these peptides might have other, subtler mechanisms of altering the pro-inflammatory response which may be applicable to *in vivo* experiments or human clinical trials. The peptide CEMA, for example, blocks some, but not all, of the transcriptional responses induced by LPS in a macrophage-like cell line and induces expression of genes believed to be involved in host responses to infection.[74] Similarly, LL-37 has been demonstrated to increase the expression of anti-inflammatory genes such as IL-10 in a macrophage cell line.[29] BPI may also have multiple mechanisms of action as it has been shown that low levels of BPI reduce pro-inflammatory cytokine production induced by LPS, but much higher concentrations of BPI are required to neutralise the anti-inflammatory cytokine IL-1RA.[49] Also, LL-37, the insect-derived peptide CEME, and the bovine cathelicidin indolicidin, can be added to a monocyte cell line up to an hour after LPS and still reduce pro-inflammatory cytokine production.[29,69] In animal models, peptides can often be added up to 6 h after LPS and still reduce pro-inflammatory cytokine production and mortality. There is increasing interest in the properties of these peptides independent of their LPS/LTA neutralising properties.

PERSPECTIVES

Despite the fact that there are many animal models for sepsis and septic shock-like conditions, it is questionable if they are truly representative of how shock manifests in humans. It has been proposed that one of the difficulties in interpolating results from animal models to human application is that these models do not reflect accurately the course of disease in humans. Table 1 summarises the animal models used in sepsis studies.

In many animal model studies, host defence peptides are not more effective in preventing mortality than conventional antibiotics and at first blush it might appear that they are no more effective than currently established therapies. However, in all cases in which they were measured, the serum levels of cytokines and LPS were reduced. In human disease, it is clear that treatment with antibiotics is not sufficient to prevent mortality, as decreases in viable bacteria do not correlate with survival. It is thus believed that the fatal aspect of septic shock is mediated by overwhelming production of pro-inflammatory cytokines. In studies where the efficacy of host defence peptides is compared with that of conventional antibiotics, it is clear that only the peptides have an ability to reduce pro-inflammatory cytokine production.

Although cost, the efficiency of production methodologies and *in vivo* stability of these peptides have been proposed to be barriers for clinical use, the largest barrier in the development of these peptides might be the dearth of animal models which accurately reflect the development of sepsis in humans, and thus are useful for predicting the true potential efficacy of these peptides in the clinic. As virtually every antimicrobial therapy in the physician's repertoire is modified or adapted from nature's design, it is likely that further studies of the anti-sepsis effects of host defence peptides mat result in the development of novel and effective therapies.

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