Endotoxin Neutralizing Peptides

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Abstract: Neutralization and sequestration of bacterial lipopolysaccharide which plays a key role in gram-negative sepsis is required to block the progression of sepsis at early stages in addition to destroying bacteria. Many of the host defense peptides which have antimicrobial activity are also able to bind to and neutralize LPS, however, these two activities do not necessarily correlate. Due to its toxicity application of polymyxin B as the prototype of LPS neutralizing peptide is limited to topical applications and extracorporeal removal of endotoxin. Development of novel endotoxin neutralizing peptides without the toxicity of polymyxin B have been based on the natural host defense peptides, fragments of LPS binding proteins and engineered peptides. Neutralization of LPS can be achieved through several different peptide fold motifs, which are reviewed in this article. Endogenous host defense peptides, fragments of endotoxin-binding proteins and synthetic anti-endotoxin peptides fold into α-helical, β-hairpin, extended and compact conformations without regular secondary structure. In animal models many of the peptides have demonstrated good *in vitro* and *in vivo* endotoxin neutralizing activity but up to now none of the peptides has been approved for clinical application with an anti-endotoxin indication. Recent developments include preparation of novel types of endotoxin neutralizing compounds such as peptides modified by lipophilic moieties and non-peptidic molecules, particularly lipopolyamines and on the other hand additional medical applications such as extracorporeal endotoxin removal, targeting to inflammation sites or endotoxoid based vaccines.

Key Words: Endotoxin, lipopolysaccharide, peptide, neutralization, secondary structure, antimicrobial, lipopeptide.

INTRODUCTION

Recognition of bacterial lipopolysaccharide (LPS) by extracellular receptors is the first step in the development of endotoxaemia. Endotoxaemia is the consequence of the effect of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and IL-6 that are induced in monocytes in response to bacterial endotoxin. Efficient response to the presence of bacteria is vital for the survival of the organism in infection; however, excessive response can induce septic shock with high mortality. Due to the threat of bacterial infection our immune system was fine-tuned with emphasis to the recognition of the presence of bacteria and rapid initiation of the immune defense system with release of proinflammatory mediators and also antimicrobial agents. Such a strong immune response to infection may nowadays, with the availability of antibiotics, not always be the most appropriate. There is a need for treatment that will blunt the exaggerated response to infection in addition to destroying the microorganisms. Medical treatment of sepsis is very difficult, particularly at late stages, due to the release of a number of different mediators that can support sepsis, variety of effects on vascular system and multiple organ failure [1]. Therefore early recognition of the presence of endotoxin in the body and neutralization and sequestration before it binds to the receptors and initiates the signaling cascade that leads towards the activation of downstream septic mediators would be the most desirable treatment. Compounds that are apt to achieve this should have high affinity for LPS and ability to neutralize it, be able to sustain high concentrations in the circulation, should not be toxic for eucaryotic cells and have other necessary pharmacological properties. LPS can bind to a number of serum proteins which present it to other cellular receptors e.g. lipopolysaccharide binding protein (LBP) or heat shock proteins (HSP) or, alternatively may remove it from the circulation (e.g. albumin, high density lipoprotein (HDL)). Affinity of LBP, CD14 and MD-2 for LPS is in the range of 10^{-8} M [2,3,74].

Some multicellular organisms such as arthropodes have evolved a number of endogenous molecules that neutralize LPS and lead to gelation as a way to achieve bacterial sequestration, while in mammals the bactericidal/permeability increasing protein (BPI) is the prominent high affinity serum endotoxin neutralizing protein. Use of proteins which could provide high affinity sequestering agents for endotoxin neutralization has a number of limitations, therefore development of other molecules with improved pharmaceutical and technological properties is desired.

Bacterial lipopolysaccharide has a number of special physicochemical properties (reviewed in the chapter by K. Brandenburg) which define the properties of their receptor binding sites and also of neutralizing molecules. At this point we have to emphasize the difference between LPS binding

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and neutralization. Many small molecules are able to bind to LPS with high affinity, however some of them neutralize LPS *in vivo* only poorly [4]. One of the reasons, common also to many antimicrobial peptides is that when the interaction is electrostatically driven it is significantly diminished in the physiological milieu. The strong hydrophobic interaction which represents an important contribution to LPS neutralizing may on the other hand lead to toxic effects on host cell membrane.

In this review we have divided endotoxin-neutralizing peptides into tree types: endogenous peptides/proteins, fragments of LPS binding proteins and designed peptides/ lipopeptides.

NATURAL ENDOTOXIN NEUTRALIZING PEPTIDES

Antimicrobial peptides are the part of the defense against microbes that has evolved in multicellular organisms [5]. Currently there are more than 500 known antimicrobial peptides (http://www.bbcm.univ.trieste.it/~tossi/antimic.html).

Relation Between LPS-Neutralizing and Antibacterial Activity

LPS neutralizing activity is often associated with antibacterial activity particularly against Gram-negative bacteria. Many peptides that neutralize LPS have been studied primarily in the context of their antimicrobial activity. These activities do not necessarily overlap although most LPS neutralizing peptides also inhibit or kill Gram-negative bacteria, while the opposite does not hold true because there are various mechanisms of antibacterial activity of peptides. Cationic antimicrobial peptides need to penetrate the outer membrane of Gram-negative bacteria, composed primarily of lipopolysaccharide, before the peptides reach the cytoplasmic membrane where the antimicrobial activity is believed to take place [6,7]. Transport across the outer membrane of Gram-negative bacteria occurs by the self-promoted uptake, in connection to the negatively charged surface of the outer membrane. The peptides either neutralize the charge over a patch of the outer membrane or bind to the divalent cation binding sites on LPS. In both cases the peptide is than able to penetrate through defects in the outer membrane [8]. Recently peptide penetration of the inner cell membrane without affecting its permeability has been shown for several peptides (e.g. buforin, lactoferricin B, magainin 2 [9,10]). Antimicrobial activity of these peptides is exerted through binding to intracellular targets such as DNA.

Combination of antimicrobial with LPS neutralization activity is a desired property for a treatment of sepsis [11]. This task can either be achieved with a single compound or by a combination of compounds with antimicrobial and endotoxin-neutralizing activity [12,13]. Endotoxin neutralizing peptides have shown synergistic effect with hydrophobic antibiotics [14]. It has been shown that small antimicrobial peptides (buforin II, indolicidin and synthetic peptide KFFKFFKFF) were effective in reduction of endotoxin and prevented TNF-α upregulation in the rat model of sepsis provoked either by injection of LPS or live bacteria [15]. Designed peptides rich in tryptophan and arginine residues have shown potent antimicrobial activity even for very short sequences, however, their endotoxin neutralizing effect has not been reported [16,17].

Small Cyclic Peptides of Microbial Origin

Polymyxin B

Polymyxin B is a small cyclic lipopeptide produced by *Bacillus polymyxa*. It neutralizes LPS and has antimicrobial activity particularly against Gram-negative bacteria. Due to its neurotoxic and nephrotoxic effects, in large part due to its slow degradation *in vivo* [18,19] it is only suitable for topical applications. Nevertheless polymyxin B is extensively used as a golden standard for benchmarking the efficiency of other compounds in neutralization of endotoxin. Polymyxin B exists in solution in rapid exchange between conformations which include type II' beta-turn from residues 5-8, and gamma-turn in residue 10. Addition of LPS induces formation of a defined conformation of polymyxin B which segregates hydrophobic and positively charged side chains [20]. Binding studies of polymyxin B to LPS monolayers by surface plasmon resonance revealed that it binds to LPS in two stages: the initial bimolecular stage, involving predominantly electrostatic interactions is followed by the second, rate determining monomolecular stage which probably represents insertion of acyl chain of polymyxin B into the lipid layer [21,22]. This second stage is probably crucial for efficient sequestration of LPS since the polymyxin B nonapeptide binds with comparable affinity to LPS but is not effective in its neutralization [19]. Molecular docking of polymyxin B with LPS, analysis of its binding with calorimetry [23] and surface plasmon resonance on LPS monolayers [21] also support the notion that hydrophobic interaction between alkyl chain of polymyxin B and lipid chains of LPS play a major role in the interaction. Association of polymyxin B with LPS disrupts its supramolecular structure [24] and the "endotoxic conformation" [25] which is also believed to be important for the physiological effects of LPS.

Peptides and Small Proteins of Vertebrates

α-helical Peptides

Magainin and Cecropin

Magainin, a small peptide, isolated from the skin of African clawed frog, has been studied primarily in the context of its antimicrobial activity. When it interacts with outer membrane of gram-negative bacteria its primary target is LPS [26], where it causes a concentration-dependent disordering of the LPS fatty acyl chains [27]. Its effect on LPS disordering was shown to correlate primarily with the charge on LPS. Upon membrane insertion (made of phospholipids or LPS) magainin forms α-helical structure [28,29].

Modification of peptides from the antisense strand of magainin indicated that the increased positive charge correlated with the affinity for LPS but also showed the importance of contiguity of the distribution of positive charges [30]. These peptides also had antimicrobial activity, disrupting both outer and inner membrane. Cecropin, an

antimicrobial cationic peptide isolated from insects binds to the lipid A moiety of LPS [31]. Hybrid between cecropin and mellitin showed improved binding to LPS and permeabilization of the outer membrane of Gram-negative bacteria [32]. In a panel of α-helical peptides produced as hybrids and by variation of natural α-helical antimicrobial peptides cecropin and mellitin the antimicrobial activity, LPS binding and cytokine production was investigated. Peptides MBI-27 and MBI-28 bind to LPS with similar affinity and suppressed production of cytokines in macrophages in extent comparable to that of polymyxin B [33]. Good correlation was observed between antimicrobial and endotoxin neutralizing activities [34].

Bactenecins

Bactenecins are a group of peptides that are synthesized as precursors with N-terminal protein domain similar to the cystatins – inhibitors of cysteine proteases [35]. The role of the propart is not clear although it also has antimicrobial activity [36]. The C-terminal peptide of cationic antimicrobial protein CAP-18 named LL-37 (residues L1 to S37) which has antimicrobial activity and neutralizes LPS, is rich in basic residues and forms α-helix similar to magainin or mellitin [37,38]. LL-37 is upregulated during the immune response. It has been shown to protect mice against endotoxaemia and to inhibit macrophage stimulation by LPS, lipoarabinomannan (LAM) and lipoteichoic acid (LTA) [39]. LL-37 also up-regulated the expression of chemokines without stimulation of the proinflammatory cytokine TNF-α. Introduction of two additional hydrophobic and furthermore of three basic residues into an 18-mer (residues 15-32) augmented LPS neutralizing activity and suppressed TNF-α production in murine model [40]. Bac7(1-35), a proline rich antimicrobial peptide was comparable to polymyxin B in its ability to neutralize LPS *in vitro*, decrease plasma endotoxin levels and decrease mortality of rats injected with *E.coli* [13].

Gramicidin

Gramicidin S is a cyclic decapeptide of bacterial origin produced by a nonribosomal synthesis. It is active primarily against Gram-negative bacteria and binds to LPS with IC50 approximately 5 fold that of polymyxin B [7], predominantly through hydrophobic interactions, since it contains only two basic residues. Gramicidin S binds equally to zwitterionic and anionic lipids. Addition of gramicidin S reduced gel-to-liquid crystalline phase transition temperature of S-type LPS [41]. Structure of gramicidin consists of a two-stranded antiparallel β-sheet with the strands interconnected by two type II β-turns.

Defensins

Defensins are small disulfide linked peptides consisting of 30-45 residues. They are produced in the granules of neutrophils but have been described also in insects and plants [42]. High number of disulfides maintains structural integrity of the fold consisting mainly of β-strands. In plants and insects they contain an additional α-helix. Six isoforms of alpha-defensin and genes coding for 28 beta-defensins have been predicted in human genome [43]. Besides having antimicrobial activity defensins also play an important role in regulating the immune response. Transcription of defensins is upregulated by LPS [44,45,46,47]. It has

magainin	**GIGKFLHSAGKFGKAFVGEIMKS**
MBI-27	**KWKLFKKIGIGAVLKVLTTGLPALIS**
LL-37	**LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES**
Bac7(1-35)	**RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP**
NK-2	**KILRGVCKKIMRTFLRRISKDILTGKK**
BPI(86-99)	**KISGKWKAQKRFLK**
LBP(86-99)	**RVQGRWKVRKSFFK**
LALF(36-45)	**CHYRIKPTFRRLKWKYKGKFWC**
MD-2(102-116)	**FSFKGIKFSKGKYK**
apo-HDL (18-mer fragment)	**DWLKAFYDKVAEKLKEAF**
MARCKS effector domain	**KKKKKRFSFKKSFKLSGFSFKKNKK**
lactoferrin (1-18)	**GRRRRSVQWCAVSQPEAT**
lactoferrin (21-31)	**FQWQRNIRKVR**
TALF(29-59)	**GHECHYRVNPTVKRLKWKYKGKFWCPSWTS**
tachyplesin I	**KWCFRVCYRGICYRRCR**
polyphemusin I I	**RRWCFRVCYRGFCYRKCR**
buforin 2	**TRSSRAGLQFPVGRVHRLLRKGNY**
SAEP-2	**KTKcKFLKKc**
SAEP-4	**KFFKFFKFF**
indolicidin	**ILPWKWPWWPWRR**

Fig. (1). Sequences of peptides of protein fragments reported to bind LPS.

ecently been reported that murine beta-defensin directly nteracts with TLR4, receptor for LPS in dendritic cells, nducing their maturation [48]. Human defensin alpha is able o neutralize LPS although approximately three orders of nagnitude weaker than BPI and is inactive against the S-type of LPS [49].

Peptides with high content of tryptophan

Indolicidin and tritrpticin are antimicrobial peptides produced by neutrophils which contain a large fraction of ryptophan residues [50]. Indolicidin binds strongly to LPS nd *in vivo* reduced lethality of endotoxin or live bacteria 15]. In micelles indolicidin forms extended structure with wo half turns and a hydrophobic core, consisting of ryptophan and proline residues [51]. Interaction with LPS is ıot stereospecific since retroindolicidin binds with comparable ıffinity to endotoxin. Binding to LPS induces a conformational adaptation of tryptophan residues [52]..

PEPTIDE FRAGMENTS OF ENDOTOXIN-BINDING PROTEINS

Extracellular Endotoxin Receptors

In vertebrates and other multicellular organisms recognition of endotoxin is performed by a cascade of extracellular receptors leading to membrane spanning Toll-like receptors as the transducers of the signal across the cell membrane [53,54]. Presence of extracellular proteins LBP, CD14 and MD-2 is essential for response to low concentrations of endotoxin. These proteins bind LPS and some of their peptide fragments are also capable of binding and neutralizing LPS. One or several peptide fragments that bind LPS have been identified in LBP [55], BPI [56,57] [58] and MD-2 [59] but not in leucine rich repeat protein CD14 which interacts with LPS in plasma. Direct interaction of TLR4 with LPS is still controversial [60].

LBP and BPI

LBP and BPI are homologous proteins composed of two domains which have similar fold as determined from the tertiary structure of BPI [61] and molecular model of LBP [62]. The two domains form an extended, boomerang-like structure, consisting mainly of β-strand. LBP and BPI are both soluble extracellular proteins although LBP intercalates in a defined orientation into negatively charged cell membrane [63]. LBP augments the activity of LPS by dispersing the LPS aggregates and extracting the monomer [64] which is presented to CD14 [65]. High concentration of LBP in the serum on the other hand protects mice from septic shock [66,67]. BPI, in contrast to LBP, binds to LPS, increases the size of the aggregate [64] and prevents its further interaction with other molecules, effectively neutralizing it. BPI is thus a true LPS-neutralizing molecule although, at high concentrations in plasma, LBP and CD14 also neutralize the endotoxin. Exchange of N- and C-terminal domains of LBP and BPI have shown that it is primarily the N-terminal domain that binds to LPS. Isolated N-terminal domain of BPI is a potent neutralizer of endotoxin [68] with therapeutic indication in meningococcal sepsis [69]. Interaction of LBP and BPI with LPS at the molecular level remains unclear despite knowledge of the tertiary structure of BPI. Crystal structure contains a phosphocholine molecule bound to the hydrophobic groove in each of the two domains [61]. Residues Arg94 and Lys95 in a β–hairpin with high proportion of basic residues are essential for LPS binding and subsequent cell activation [66]. These residues, however, are separated from the bound lipid by almost 50 Å. Peptide scanning of BPI indicated the same hairpin region as the most potent for endotoxin neutralization [58,57]. Peptides from the corresponding region of LBP inhibited macrophage secretion of TNF-α after stimulation by LPS, prevented binding of LPS to LBP and also possessed bactericidal activity [70,56]. The minimal fragment essential for LPS binding was identified as the region 86-99, with

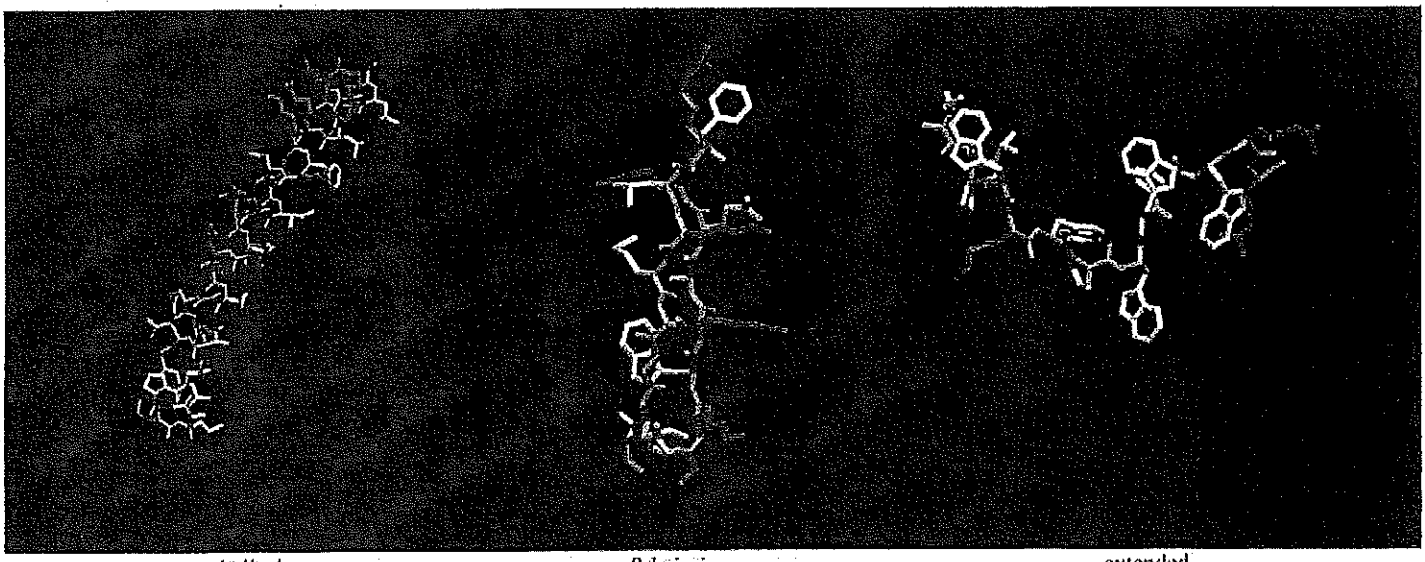

Fig. (2). Representative 3D structures of peptides that interact with LPS. Residues are colored according to type (white-hydrophobic, blue-basic), backbone is colored in gold. Structures were rendered with program VMD [153].

residues Trp91 and Lys92 as essential, while mutation of Arg94, Lys95 and Phe98 to alanine increased the inhibition of LPS binding to LBP [55]. Enhancement of β–turn propensity of the peptide based on BPI improved LPS neutralization *in vitro* and in murine model of endotoxaemia [71]. The most potent of synthetic peptides derived from BPI/LBP was a cyclic hybrid peptide, composed of portions from LALF and LBP [72] which was effective in mice when administered up to 7 hours after LPS injection.

MD-2

MD-2 associates tightly to the extracellular domain of TLR4 essential for cellular response to low concentrations of LPS [73]. It has been shown that MD-2 binds LPS with an apparent K_D of 65 nM [74]. A peptide fragment with high content of basic residues in addition to high hydrophobic moment, similar to other LPS-binding peptides, has been identified within the MD-2 sequence. This peptide neutralizes LPS in the LAL assay and has weak antimicrobial activity [59]. NMR study displayed strong signal due to the interaction between MD-2 peptide and LPS as well as with LTA, but not with peptidoglycan. Homology modeling of tertiary structure of MD-2 based on GM2 activating protein and Derp2 indicate that the LPS binding epitope on MD2 is discontinuous and will unlikely lead towards effective LPS-neutralizing peptides (R.Jerala, unpublished).

PEPTIDE FRAGMENTS OF PROTEINS OF THE SAPOSIN FAMILY

Saposin domains occur in many membranolytic proteins, such as acyloxyacyl hydrolase which can detoxify LPS by deacylation [75], acid sphingomyelinase, granulysin, NK-lysin and surfactant active proteins and amoebapores [76,77]. "Saposin fold" consists of five α-helices and is characterized by three disulfide bonds [78,79]. NK-lysin and granulysin are active against a wide range of bacteria as well as against tumor cells [80]. They are believed to kill bacteria by forming pores in bacterial membrane [81,82,83,84], proposed to be formed not by membrane spanning helices but rather by molecular electroporation [85]. Peptide from the third α-helix of NK-lysin retains high antimicrobial activity [85]. Peptide NK-2 from the same region has potent activity against *Trypanosoma cruzi* [86] and discriminates between the phosphatidylethanolamine and phosphatidyl-choline [87]. Peptide comprising helix-loop-helix motif from helices 2 and 3 had better activity against mycobacteria than integral NK-lysin and is also active against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [88]. NK-lysin and its peptide fragment interacts with endotoxin [89, 155] and competes with polymyxin B for binding to lipid A moiety of LPS suggesting that it may be endogenous LPS binding protein similar to the surfactant protein B which protects against endotoxin-induced inflammation of lung [90].

Peptide Fragments of other Vertebrate Proteins that Bind LPS

High-density lipoproteins (HDL), albumin, low-density lipoproteins (LDL), serum amyloid P component and several other serum proteins also bind LPS [91]. High density lipoprotein (HDL) aids in detoxification of LPS in plasma by binding a portion of endotoxin [66] and is important for the interaction of LPS with LBP. HDL can neutralize endotoxin *in vitro* and *in vivo*. Capacity of lipoproteins for endotoxin far exceeds typical LPS concentrations in sepsis and 60% of LPS in blood bound to lipopoproteins is associated with HDL [92]. Transgenic mice with 2-fold elevated level of HDL had improved survival rate in endotoxicosis [93]. Binding of HDL to LPS induced the change in LPS supramolecular structure from unilamellar/inverted cubic into a multilamellar structure [94]. Reconstituted HDL from either HDL or synthetic 18-mer derived from apolipoprotein A-I with phospholipids, but not apo-HDL was able to protect mice from endotoxin, presumably through the insertion of LPS into the reconstituted HDL [93]. ApoE can also bind LPS and redirect it from macrophages to hepatocytes. ApoE deficient mice are more sensitive towards LPS than wild type [95] and recombinant apoE may be used for neutralization of LPS.

Buforin 2, is produced by the proteolytic degradation of histone. It has the ability to penetrate the bacterial membrane and DNA has been proposed as its antimicrobial target [96]. Peptide folds into an amphipathic α-helix distorted by a kink at Pro11 residue [97]. *In vivo* buforin decreased the level of endotoxin in septic shock animal models [13,15,98]. Peptides from the C-terminal region of histones H1, H2A, H2B, H3, and H4 are also able to bind LPS wit affinities higher than of polymyxin B and were able to repress the activation of macrophages by LPS [99].

Heparin binding protein, also known as azurocidin or cationic antimicrobial protein of 37 kDa (CAP37) is a multifunctional protein, present in granules of neutrophils with important role in inflammation [100]. It kills gram-negative bacteria, regulates function of macrophages/ monocytes and binds endotoxin. Based on the structural fold it belongs to the family of serine proteases but has no enzymatic activity. Bactericidal and endotoxin-binding domain of CAP37 has been proposed [101]. Most of the peptide fragment with antimicrobial and endotoxin-neutralizing activity is not accessible to the solvent and thus can not represent the endotoxin-binding region of the native protein [101]. Mutagenesis of eight basic residues located in discontinuous epitopes on loops 3 and 4 which were selected based on the tertiary structure of CAP37 [102] revealed that these residues are responsible both for heparin binding and bactericidal activity against gram-negative bacteria [103].

Serum amyloidal P (SAP) component was found to bind to LPS and prevent its binding to monocytes, however, it was not able to prevent binding of LPS to soluble CD14. Synthetic peptide derived from SAP (27-39) was able to antagonize the effects of LPS in human blood [104]. This peptide can, however, similarly as the previously mentioned peptide of CAP37 not be responsible for the LPS binding of the SAP since it is buried in the core of the folded protein and could not interact with LPS under native conditions.

PEPTIDES BASED ON MARCKS FAMILY OF PROTEINS

MARCKS family of proteins (myristoylated alanine rich protein kinase C substrate) consists of two representatives found in vertebrates: MARCKS and MARCKS-related

protein (MRP) [105]. They do not have a persistent tertiary structure and are characterized by the unusual aminoacid composition, where 85% of the residues consists of only six aminoacid types. MARCKS is present at high concentration in brain and is significantly upregulated by stimulation with LPS [106]. MARCKS binds to calmodulin/Ca, PIP_2 and actin [107,108,109]. These interactions are mostly mediated through the effector domain, located in the middle of the sequence. This effector domain is extremely rich in basic residues, while the whole protein has a net negative charge. Aromatic residues are located exclusively within this effector domain. Interaction of MARCKS with its binding partners is regulated through phosphorylation and proteolysis [110]. MARCKS acts as a mediator between several signal transduction pathways [111]. Recently it has been demonstrated that MARCKS and MRP bind to LPS and that the effector domain prevents secretion of TNFα in macrophages stimulated by LPS. LPS binding site has been localized to the heptapeptide region RKFSFKK which overlaps with actin binding site of MARCKS with the consequence that MARCKS effector domain depolymerizes actin (Mančck *et al.*, submitted manuscript).

LACTOFERRIN

Lactoferrin is an iron binding glycoprotein present in several mucosal secretions such as milk and saliva as well as in granules of neutrophils [112]. Two LPS-binding sites have been identified on the molecule – high affinity site at the N-terminus (Kd ~ 4 nM) and low affinity site (Kd ~ 400 nM) in the C-terminal domain [113]. Proteolytic digestion e.g. with pepsin in stomach releases a peptide fragment, called lactoferricin [114]. This peptide has even stronger antimicrobial and LPS-neutralizing activity than integral protein. Separation of the peptide from the rest of the protein results in the conformational transition from α-helix to β-hairpin, stabilized by a disulfide bond, although the antimicrobial activity does not depend on the presence of a disulfide [115]. Small hexapeptide core RRWQWR of lactoferrin folds into a defined structure with hydrophobic core in the presence of SDS-micelles [116]. Bovine lactoferricin has more potent antimicrobial activity than human homologue due to the higher proportion of tryptophan residues [117]. In addition to this region of lactoferrin (17-41), the N-terminal 33-residue fragment which contains a stretch of basic residues, has strong antibacterial and endotoxin neutralizing activity. Coinjection of N-terminal lactoferrin peptide with LPS reduced the lethality in mice although the neutralization capacity was diminished by serum [118]. Recent NMR studies of linear human peptide fragment of lactoferrin reveal that in complex with LPS the peptide folds into a defined structure, guided by the interaction between basic residues of the peptide and phosphate groups of LPS and the segregation of hydrophobic groups in lactoferricin (Japelj *et al.*, manuscript in preparation).

PEPTIDES FROM LPS-BINDING PROTEINS OF HORSESHOE CRAB

Many multicellular organisms besides vertebrates have a system of defense against bacterial infection. In arthropods the presence of bacteria triggers a clotting reaction, based on the proteolytic cascade [119]. Receptors for LPS present in the haemolymph of horseshoe crab form tight complex with LPS and are the basis for analytical LPS detection. Peptides from several of the LPS binding proteins also neutralize LPS. The most extensively studied has been the limulus antilipopolysaccaride factor (LALF), also called endotoxin neutralizing protein (ENP), with known tertiary structure [120]. The amphiphillic β-hairpin in this structure presumably binds LPS. Linear peptides from this region bind LPS weakly but if the peptides comprising the core residues 36-45 are cyclised by a disulfide bond their LPS neutralization ability become comparable to polymyxin B [121]. The conformation of this loop in LALF superposes well with the β-hairpin from LBP and BPI with similar pattern of basic and hydrophobic residues. However, the overlap is the best in the reversed orientation which explains why the retro peptide of BPI has better activity than the original sequence [122]. Interchange of BPI and LALF binding loop into the LBP retained the LPS binding ability of LBP indicating the functional similarity of these loops [123]. While the LALF (31-52) peptide inhibited induction of TNF-α it did not affect NO generation [124], indicating that peptides may modify the cellular response to endotoxin. Peptide from *Tachypleus* antilipopolysaccharide (LPS) factor (TALF) – TALF(29-59) decreased induction of cytokines from human monocytes, and LPS-induced lethality in sensitized mice. The minimal LPS binding sequence consists of the peptide 41-53 [125].

Other peptides based on LPS-binding peptides of horseshoe crab originate from sushi domains of factor C and have the affinity constants for LPS between 10^{-9} and 10^{-10} M. These domains are related to the complement system. In S1 domain cooperativity increases affinity for three orders of magnitude, while binding of S3 is noncooperative. Core of the LPS recognition region consists of 34-residue peptide fragments, containing a disulfide bond [126]. Upon binding to LPS circular dichroism spectra indicate conformational transition from random coil to α-helix and β-structure for S1 and S3 peptide, respectively [126].

Tachyplesin I from horseshoe crab is a small peptide containing 2 disulfide bonds which are essential for its specific LPS binding [127]. In comparison to magainin which does not discriminate between binding to acidic phospholipids and LPS, tachyplesin I is highly specific with 280-fold difference in partition coefficient between LPS and phosphatidyl glycerol. This specificity is reduced by the removal of disulfide bond. CD spectra of the homologous peptide from horseshoe crab polyphemusin I indicate the presence of the type II β-turn-rich structure in aqueous solvent and conformational transition to β-sheet structure in anionic lipid environment. Binding to LPS correlates well with the ability to inhibit LPS-induced production of TNF-α and IL-6 in macrophages. Variants with modifications in loop regions which were predicted to have increased amphipathicity had improved antimicrobial activities in animal models of infection and endotoxaemia [128].

SYNTHETIC ANTI-ENDOTOXIN PEPTIDES (SAEP)

Sequential and structural pattern of LPS-binding peptides has been identified based on the sequence comparison of endogenous and microbial LPS-neutralizing peptides and

based on 3D structures of peptides and proteins. This led to the design of synthetic anti-endotoxin binding peptides, with the purpose of identifying the structural requirements for strong affinity and improvement of the pharmacological properties such as bioavailability or toxicity towards eucaryotic cells. One of the principles for designing new endotoxin neutralizing peptides was based on engineered β–sheet peptides [129]. It was found that the β–sheet conformation of peptides correlates well with capacity to neutralize LPS, but not with bactericidal activity. The most active SAEPs were 50-100 times weaker than peptides based on the BPI. The design of β–turn peptides was also the basis for cyclic peptides specific for Gram-negative bacteria [130].

Synthetic Anti-Endotoxin Peptides Mimicking the Structure of Polymyxin B

The structural characteristics of the peptide antibiotic polymyxin B have led to investigation of the minimal and optimal requirements of peptide structures for binding to the Lipid A moiety of LPS [12]. Particularly, the role played in the structure of polymyxin B by the cationic α,γ-diamino butyric acid (DAB) residues and the cyclic conformation of the molecule were considered. Accordingly, several structures of synthetic peptides were designed following the concept of amino acid replacement on the basis of molecular mimicry. In these synthetic structures DAB was replaced by Lysine residues, since this natural amino acid is a homologue of DAB; the amino acid D-phenylalanine (toxic to mammalian cells) was replaced by the natural isomer L-Phenylalanine; the cyclic rearrangement of the peptide structure was achieved by strategically inserting, in the primary sequence, two cysteine residues which, following oxidation, have resulted in the disulphide bridge compound, similarly to the one present in the structure of natural proteins. Using a library of peptide analogs, basic principles could be drawn on the requirements for binding and detoxification of the lipid A moiety of bacterial LPS.

These principles refer to the minimal and optimal size of an amino acid sequence and the relevance of its secondary rearrangement; the appropriate balance between cationic and hydrophobic amino acids and their position in the sequence; the orientation of the peptide-bonds of the sequence; the size of the disulphide-driven cycle. All these factors have been useful in understanding and defining the minimal and optimal features of a peptide structure able to bind and detoxify Lipid A [12,131,132]. A summary of these principles are consistent with optimal peptide sequences containing between ten and twelve amino acid residues, rearranged in cyclic conformation, having an index ratio (cationic / hydrophobic amino acids) equal to or higher than one. Two main steps have been identified in the binding of SAEP to the lipid A moiety of LPS a preliminary charge-to-charge interaction between the cationic amino acids of the peptide sequence with the phosphate groups of the Lipid A moiety and a final, hydrophobically-stabilized binding, occurring between the hydrophobic amino acids of the peptide sequence and the alkyl chains of the lipid A moiety [12]. Once formed, the SAEP-Lipid A / LPS complex is stable in a broad range of pH and ionic-strength.

It is often not safe to infer the *in vivo* activity based on *in vitro* measurements. For instance, on the basis of an *in vitro* parameter like the affinity constant value of a peptide for lipid A [12], no safe predictions can be done on the efficiency of the resulting detoxification activity *in vivo*. The main reason for this observation relies on several additional parameters to be considered *in vivo*, like the stability of the peptide structure to serine proteases of mammalians; the balance of the peptide distribution between blood and target organs; the capability of the peptide to effectively compete with the LPS- receptor(s); the clearance-period of the peptide from the bloodstream. All these factors, affect the final efficiency of given peptide structure in the detoxification of LPS *in vivo*. These considerations explain why, despite a molar stoichiometry of binding *in vitro* between an optimal peptide structure and Lipid A [12], there is the need of providing a significant excess of the peptide in binding experiments of LPS with cells expressing CD14 receptor as well as *in vivo* when effectively controlling the toxicity of LPS [133].

LIPOPEPTIDES AND LIPOPOLYAMINES

Polymyxin B as the prototype endotoxin neutralizing peptide is a cyclic lipopeptide. The alkyl moiety is essential for the neutralizing activity as well as for antimicrobial activity. Nonapeptide – a polymyxin B, produced by cleavage of a dipeptide with alkyl chain, retains its binding ability to LPS but does not block its interaction with other cellular receptors and does not have antibiotic activity [19]. Colistin nonapeptide also neutralized LPS released from bacteria very little in comparison to its parent lipopeptide [134]. However, these observations were not confirmed in the studies with SAEP which do not contain the alkyl moiety and have shown comparable detoxification activity to polymyxin B *in vitro* as well as *in vivo* [12,132,135].

Addition of alkyl chains to other cationic peptides led to the significant improvement of their LPS neutralization capacity as well as to the augmentation of their antimicrobial activity. Addition of alkyl chains to other peptides resulted in improvement of LPS-neutralizing activity, depending on the type of hydrophobic moiety added. Alkylated peptide fragments of bovine lactoferrin demonstrated improvement of antimicrobial activity against Gram-negative bacteria [136]. Human fragment of lactoferrin which has weak antimicrobial activity, demonstrated the maximal increase in its LPS-neutralization and antimicrobial activity by the addition of C12 alkyl chain at its C-terminus. This has been demonstrated for peptide fragments of lactoferrin, where the addition of an alkyl chain with 12 carbon units increased the neutralization of endotoxin in a LAL test 10 fold, with an improvement of antimicrobial activity increased most significantly for Gram-positive bacteria [137].

Importance of matching the distance between cationic groups of a ligand and phosphate groups of the lipid A moiety was demonstrated by binding studies of a series of diamino compounds [138]. Strong binding of several small amphiphilic organic compounds, such as pentamidine or chlorhexidine with appropriate distance between the basic groups to LPS has been demonstrated, however, their efficiency of LPS neutralization was negligible *in vivo*

[138,4]. It was concluded that the paucity of hydrophobic interactions was responsible for this discrepancy.

The notion that the salient features of peptide neutralization of LPS are due to positive charge and amphipathicity were corroborated by lipopolyamines which represent the molecular simplification to these basic principles. Reduction of structural elements of lipopeptides to functional groups containing both positive charge and hydrophobic moiety results in lipopolyamines. Lipopolyamines DOSPER and DOSPA that are used for DNA transfection of eucaryotic cells showed *in vitro* binding of LPS and protection of mice against the effects of endotoxin [139,140]. These nontoxic compounds neutralized LPS *in vitro* with a Kd of 4.9 μM which is 1/10 of the affinity of polymyxin B, however, they could still demonstrate protection of mice at administration of lethal dose of LPS [139]. DOSPER itself was ineffective for treatment of bacteremia but in combination with antibiotic provided protection of mice infected with *Pseudomonas aeruginosa* [141].

PERSPECTIVES FOR CLINICAL STUDIES ON SAEP

In the clinical use of a peptide structure addressed to the prophylaxis or therapy of a pathology which involves LPS and its endotoxic effects, one must take in consideration several factors affecting the treatment. Among others, safety issues. These are related to the controlled biodegradability of the peptide structure, in order to avoid accumulation phenomena in target organs, like those clinically well known for polymyxin B which accumulates in the kidneys [142]. There are two reasons leading to the toxicity of polymyxin B in these target organs: the lack of biodegradability of its structure, refractory to serine proteases of mammalians and interactions of this drug with the epithelium of the kidneys so that it cannot be eliminated in the urine. SAEP have been purposely designed to avoid the toxicity of polymyxin B, being slowly degraded by serine proteases, while retaining the detoxification activity of the drug. Because the relatively high dose of SAEP to be administered in acute and chronic treatment, toxicology studies on SAEP in animal models (mice, rabbits and beagle dogs) have considered doses of SAEP in the order of a few mg / kg , with no significant side effects detected systemically as well as in target organs (M. Porro, personal observations).

There are at least two human pathologies reported to be significantly related, directly or indirectly, to the endotoxic activity of bacterial LPS : endotoxaemia detected in fulminant meningitis due to acute infections of *Neisseria meningitidis* and endotoxaemia related to septic shock. While the latter is a pathology where the role of LPS is still under investigation, there are few doubts that the high levels of meningococcal LPS detected in fulminant meningococcemia, resulting in the massive release of pro-inflammatory cytokines, are responsible for the fatal outcome of the former pathology [143]. According to these observations, BiosYnth Srl is now developing a candidate vaccine which targets *Neisseria meningitidis* LPS. This vaccine is prepared using highly purified LPS, from pathogenic strains of *Neisseria meningitidis* which is detoxified by SAEP through the formation of a complex SAEP-LPS [144]. This complex, named Endotoxoid, has been shown in preclinical studies to be safe, having a reduction of toxicity equivalent to 3.0 logs with respect to the native homologous LPS, and immunogenic by inducing LPS-specific IgG antibodies that bind to the antigen on the surface of the bacterium, fix the complement cascade, kill the bacterium by complement-mediated activity and also neutralize significantly the toxicity of LPS in the bloodstream. This Endotoxoid-based vaccine against *Neisseria meningitidis* is now ready to enter Phase I and Phase II clinical trials. If successful, the Endotoxoid concept will then open the door to the prophylaxis of infectious diseases like typhoid, shigellosis, cholera and other significant Gram-negative bacteria.

There is clearly the need of a more accurate clinical definition of the pathology, specifically addressed to the identification of the leading cause(s) responsible for it. The role of LPS as a factor determining the outcome of most of the patients affected by septic shock is not clear, even though endotoxemic effects due to LPS in the bloodstream of patients are reported. Also, due to the very narrow time-frame within which a treatment can be attempted on the patient, there is a significant margin of uncertainty on the efficacy of a treatment which considers only a single target factor. Accordingly, multiple and synergistic therapeutic treatments can be envisioned, like a combination of antibiotic and anti-LPS treatment paralleled by a treatment against pro-inflammatory cytokine release [145] and eventually removal of LPS from bloodstream using solid matrices supporting covalently-bound LPS-binding peptides [146].

A paradox that one faces in this pathology is due to the observation that the concomitant administration of antibiotic drugs may result in the increased release of LPS from the outer membrane of the infecting bacterial strain, therefore negatively affecting endotoxaemia and the resulting massive release of pro-inflammatory cytokines [147].

One of the important approaches in neutralizing the endotoxin in sepsis is extracorporeal removal of endotoxin by hemoperfusion using adsorbents that bind endotoxin [148,149]. Problems in the current types of sorbents is their biocompatibility and adsorption efficiency [150]. Besides charcoal and simple cationic compounds such as polyethyleneimine, heparin, cationicaly modified cellulose polymyxin B has also been used for selective removal of endotoxin [151]. Additional potential application may be based on the targeting of endotoxin-binding peptides to the site of inflammation, either for diagnostic purposes or for the delivery or drugs [152].

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