Endotoxin in Health and Disease

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LPS/Lipid A–Binding Synthetic Peptides

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THE PUTATIVE LIPID A–BINDING SITE AND ITS RECOGNITION BY PEPTIDE STRUCTURES

The amphipathic structure of bacterial endotoxins (lipopolysaccharides, LPS) relates to the supramolecular architecture of lipid A, the biologically active moiety of LPS (1). Lipid A is structurally conserved among LPS from different species of gram-negative bacteria and results from the association of several glycolipid monomers of general structure *N,O*-acyl-β-1,6-D-glucosamine disaccharide 1,4′-bisphosphate. In an aqueous environment, the glycolipid monomers form micelles according to the critical micellar concentration (CMC) of the system (1,2). The biological activity of LPS is expressed through the interaction of the lipid A component with a variety of cell- and serum-binding proteins/receptors inducing the immune system to react by secretion of proinflammatory cytokines, a process that often leads to very severe biological effects (3,4). The interaction of LPS, via lipid A, with mammalian receptor proteins appears to be a remarkably efficient process, since it is today well recognized that LPS interacts with a variety of serum proteins, with specialized cells of the immune system, and with tissues of different key organs like liver, lungs, and spleen.

During the past few years, our laboratories have undertaken studies designed to determine the thermodynamic efficiency of the interaction between the lipid A moiety of R(rough)- and S(smooth)-chemotype LPS with well-defined peptide molecules. Our primary objectives have been to characterize in detail the physical-chemical characteristics of the putative binding site expressed by lipid A, which, in turn, would be of value in defining the corresponding features required by the amino acid sequences of mammalian LPS-binding/receptor proteins with which LPS interacts. The focus of our study has been based on two fundamental observations reported several years ago and today widely recognized:

1. The capability of the peptide antibiotic polymyxin B (PMXB) to bind with high affinity to LPS and to reduce its toxicity and improve survival of mice challenged systemically with purified LPS (5)
2. The molecular explanation for the protective activity of PMXB, based on the stoichiometric interaction of the peptide antibiotic with the lipid A moiety of LPS (6), demonstrating for the first time that lipid A contains a saturable binding site.

FEATURES OF PEPTIDE STRUCTURES RECOGNIZED BY LIPID A

The antibiotic PMXB is a cyclic cationic peptide, which is composed of 10 amino acids and a hydrophobic tail, a heptanoyl/octanoyl chain, at the N-terminus of the molecule. PMXB contains in its structure several uncommon amino acids not normally found in mammalian cells, like D-phenylalanine and α,γ-L-diaminobutyric acid (DAB, six residues accounting for about 50% of the molecular mass of PMXB). DAB is a homolog of L-lysine, and the available evidence would

suggest that it is responsible for the secondary rearrangement of this cyclic peptide through the internal condensation of a DAB residue with L-threonine. It is also thought to be responsible for the proteolytic stability of PMXB to serine proteases, such as trypsin and chymotrypsin. Also of interest from the toxicological point of view, as a free amino acid it can function to replace L-lysine in the protein synthesis of mammalian cells (7). This lack of biodegradability results in the accumulation of PMXB in target organs (mainly kidneys and tissues of the nervous system) following its administration to experimental animals leading to a significant toxicity, which often ends with organ failure.

In order to investigate the contribution of the different amino acid residues in PMXB to the physical-chemical characteristics of the cyclic peptide for binding and detoxifying the lipid A moiety of LPS, we have synthesized a series of synthetic peptides containing natural amino acids, each of which replaces one of the residues found in the original structure of PMXB (8). For the primary structure of synthetic peptides, L-lysine was used for replacement of DAB residues in PMXB, L-phenylalanine for replacement of D-phenylalanine, and L-isoleucine for replacement of the heptanoyl/octanoyl chain at the N-terminal side of the molecule. Recreation of the secondary rearrangement of the peptides was achieved by insertion of two L-cysteine residues in the primary structure, followed by oxidation of the two sulfhydryl groups to form an internal disulfide bridge, thereby generating the cystine disulfide. In this case, a cystine residue was replacing the DAB-L-threonine internal bridge of PMXB, providing a natural rearrangement for the synthetic peptide (Fig. 1) in a way similar to that by which proteins naturally develop secondary structure. The primary structure of

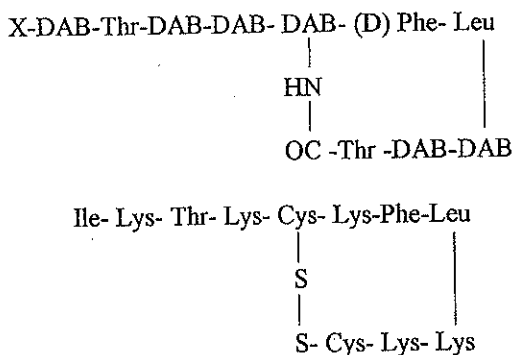

Fig. 1 Structure of polymyxin B and one of its peptide analogs. Amino acids are in three-letter code. X = 6-Methyl heptanoyl/octanoyl; DAB = α,γ-diaminobutyric acid. (From Ref. 8.)

PMXB and one of its synthetic analogs are indicated in Figure 1.

Synthetic peptides prepared according to this strategy have been of considerable value in allowing us to elucidate the molecular constraints on amino acid sequences required for efficient thermodynamically stable binding of such peptides to lipid A. Analysis of these peptides binding to lipid A has also led to the definition of the size of its binding site. Further, this approach has provided important information on some of the molecular characteristics that mammalian receptor proteins should have for potential recognition of lipid A, a crucial step in the design of peptide-based antagonists of LPS (8). Accordingly, the biological activity of synthetic antiendotoxin peptides (SAEP), as assessed by their ability to inhibit the toxic properties of a variety of R- and S-chemotype LPS, has been determined in both in vitro and in vivo studies by measuring the competitive (dose-related) inhibition of LPS-induced clotting of Limulus amebocyte lysate (LAL), local and systemic TNF and IL-6 production in mice, hemorrhagic dermonecrosis in the rabbit (local Schwartzman reaction), and lethality in mice (8,9). The results of these studies are reviewed in the following paragraphs.

GENERAL STRUCTURE OF LIPID A–BINDING PEPTIDES

Analysis of the primary as well as secondary structural features of SAEP in relation to PMXB has revealed that the characteristics of peptide-based structures required for thermodynamically efficient binding and detoxification of the lipid A moiety of LPS reside primarily in their cationicity and amphipathicity, with a relatively less, but nevertheless significant impact of the secondary rearrangement of the sequences for improving the overall affinity of binding of peptides to lipid A. In aqueous solutions, binding to lipid A appears to involve a dual-step process, through a preliminary interaction of the cationic amino acids (lysine, arginine) with the anionic groups of lipid A (phosphates), followed by the stabilization of the resulting molecular complex through hydrophobic interactions involving the fatty acid residues of lipid A, the hydrophobic amino acids (e.g., phenylalanine and leucine), and most likely the alkyl chain of lysine or arginine residues as well. These conclusions derive from experimental observations showing that anionic amino acids cannot replace cationic ones in the sequences of SAEP and still manifest high-affinity lipid A binding, that the complex between SAEP and LPS is stable over a broad range of pH

;s are ind2–11) and ionic strength (0.10–0.5 M), that the
ıry amino groups of lysine residues are still ac-
ng to this›le for reaction with specific reagents following
ı allowinglex formation, and that the complex with LPS can
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ıg paragrapl ≥1.5 kcal/mole (lysine and arginine) and hy-
ıbic amino acids with solvent parameter values
5 kcal/mole (tryptophan, phenylalanine, tyrosine,
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t the compleulus endotoxin-binding protein-protease inhib-
a broad ran each protein we have identified at least one
candidate amino acid sequence fulfilling all requirements of the algorithm defined above, and the corresponding synthetic peptides have shown the capability to bind and detoxify LPS in various assays (12–14). Comparable results have been reported for some of these protein sequences in independent studies carried out in other laboratories (15–19). In Table 1, selectivity expresses the ratio between the affinity constant value of the considered peptide for *E. coli* B5 LPS and that of polymyxin B, as detected in the competition assay described in Ref. 8. LAL inhibition indicates the minimal ratio of peptide to LPS (w/w) capable of inhibiting the clotting induced by 0.125 EU/ml (0.04 ng/ml) of *E. coli* B5 LPS, in the Limulus amebocyte lysate assay.

SEARCH FOR LIPID A–BINDING PEPTIDES IN THE PRIMARY STRUCTURE OF NATURAL LPS-BINDING POLYPEPTIDES

Several natural cationic polypeptides of the animal kingdom are known to have antibiotic properties against gram-negative bacteria, at least in part by increasing the permeability of the outer membrane (20). Among these are defensins and defensin-related peptides recently found in human skin, magainins, cecropins, melittins, tachyplesins, and rabbit cationic proteins (20–24). The algorithm has, therefore, been applied to these natural polypeptides in order to identify specific sequences as potential binding site for LPS and, if so, their potential use as synthetic anti-LPS antagonist molecules. Indeed, several amino acid sequences with cationic amphipathic characteristics were predicted in the primary sequence of these polypeptide antibiotics (25), and several of these are now under investigation in our laboratories for possible LPS-neutralizing capability. Since natural cationic antibiotic polypeptides seem to contain amino acid sequences with characteristics comparable to those required for binding and neutralizing lipid A, our attention has been also directed toward the investigation of associated complementary activities of these anti-LPS synthetic peptides; that is, the potential antibiotic activity of synthetic peptides designed de novo according to the sequences predicted by the algorithm on gram-negative bacteria (Tables 2 and 3). We have found that several of these synthetic peptides manifest direct antibiotic activity in vitro, as well as synergistic activity with hydrophobic antibiotics like rifampin and erythromycin (26).

(pH 2–11) and ionic strength (0.10–0.5 M), that the primary amino groups of lysine residues are still accessible for reaction with specific reagents following complex formation, and that the complex with LPS can be readily dissociated by 1% (w/v) SDS treatment. The length of peptide-based structures that would optimally possess these features was found to be about 10 amino acids. No detectable lipid A–binding activity was observed for sequences having less than 6–7 amino acids (8). Studies of molecular mechanics based on NMR spectra of SAEP (10) and of an analog peptide in complex with LPS have confirmed the conclusions reported above concerning the binding process in aqueous/polar solvents (11).

On the basis of these fundamental observations of peptide-LPS–binding interactions, an algorithm has been developed in order to search for potential amino acid sequences within the primary structure of well-documented LPS-binding proteins present in nature. The algorithm predicts that any cyclic or linear sequence encompassing a minimum of 6–7 amino acid residues, respectively, containing a minimum of three aliphatic cationic amino acids with solvent parameter values ≥1.5 kcal/mole (lysine and arginine) and hydrophobic amino acids with solvent parameter values ≥ −1.5 kcal/mole (tryptophan, phenylalanine, tyrosine, leucine, isoleucine, and valine), and characterized by a value of the ratio of number of cationic to number of hydrophobic amino acids (Rc/h) ≥1 has a significantly increased probability of binding with high affinity to the lipid A–binding site of LPS (12).

To test the hypothesis, we have analyzed the primary amino acid structure of well-established LPS-binding proteins to attempt to identify amino acid sequences fulfilling the three requirements of the algorithm defined above. In situations where the candidate anti-LPS sequences have been identified, the corresponding peptides were synthesized and employed in a variety of assays designed to assess binding to lipid A and neutralization of the toxic effects of LPS both in vitro and in vivo. The first set of LPS-binding proteins that were analyzed include phylogenetically different proteins reported to be involved in modulating different biological functions of LPS (Table 1). These include CD14 (the 55 kDa LPS receptor expressed on the cell membrane of some immunocompetent cells), LBP (a 60 kDa LPS-binding protein present in serum), BPI (a 55 kDa bactericidal permeability-increasing protein first identified in polymorphonuclear cell granules), LALF (a 15 kDa factor of Limulus amebocyte), and LEBP-PI (the 12 kDa limulus endotoxin-binding protein-protease inhibitor). In each protein we have identified at least one candidate amino acid sequence fulfilling all requirements of the algorithm defined above, and the corresponding synthetic peptides have shown the capability to bind and detoxify LPS in various assays (12–14). Comparable results have been reported for some of these protein sequences in independent studies carried out in other laboratories (15–19). In Table 1, selectivity expresses the ratio between the affinity constant value of the considered peptide for *E. coli* B5 LPS and that of polymyxin B, as detected in the competition assay described in Ref. 8. LAL inhibition indicates the minimal ratio of peptide to LPS (w/w) capable of inhibiting the clotting induced by 0.125 EU/ml (0.04 ng/ml) of *E. coli* B5 LPS, in the Limulus amebocyte lysate assay.

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Several natural cationic polypeptides of the animal kingdom are known to have antibiotic properties against gram-negative bacteria, at least in part by increasing the permeability of the outer membrane (20). Among these are defensins and defensin-related peptides recently found in human skin, magainins, cecropins, melittins, tachyplesins, and rabbit cationic proteins (20–24). The algorithm has, therefore, been applied to these natural polypeptides in order to identify specific sequences as potential binding site for LPS and, if so, their potential use as synthetic anti-LPS antagonist molecules. Indeed, several amino acid sequences with cationic amphipathic characteristics were predicted in the primary sequence of these polypeptide antibiotics (25), and several of these are now under investigation in our laboratories for possible LPS-neutralizing capability. Since natural cationic antibiotic polypeptides seem to contain amino acid sequences with characteristics comparable to those required for binding and neutralizing lipid A, our attention has been also directed toward the investigation of associated complementary activities of these anti-LPS synthetic peptides; that is, the potential antibiotic activity of synthetic peptides designed de novo according to the sequences predicted by the algorithm on gram-negative bacteria (Tables 2 and 3). We have found that several of these synthetic peptides manifest direct antibiotic activity in vitro, as well as synergistic activity with hydrophobic antibiotics like rifampin and erythromycin (26).

Table 1 Characteristics of Synthetic Peptides Predicted in the Primary Amino Acid Sequences of LPS-Binding Natural Polypeptides

Peptide sequences	Rc/h	Selectivity	LAL inhibition (w/w)
Predicted			
$CD14_{67-75}$ Human VKALRVRRL	1.00	0.02	100
$CD14_{68-78}$ Mouse KSLSLKRLTVR	1.00	0.03	200
LBP_{92-100} Human KVRKSFFKL	1.00	0.07	100
$LBP_{376-384}$ Human FLKPGKVKV	0.75	0.04	100
BPI_{27-34} Human KELKRIKI	1.33	0.04	100
BPI_{90-99} Human KWKAQKRFLK	1.67	0.20	10
$LALF_{41-51}$ Crab KRLKWKYKGKF	1.50	1.83	1
LEBP-PI_{5-17} Crab CQSWKSSEIRCGK [S — S]	1.50	0.05	100
Control			
LEBP-PI_{86-98} Crab CRQHGTYINCLHV [S — S]	0.25	0.00	>1000
$BPI_{153-160}$ Human IQLPHKKI	0.67	0.00	>1000
$LALF_{33-41}$ Crab HYRINPTVK	0.67	0.00	>1000

Amino acids appear in one-letter code. Rc/h value defines the ratio between aliphatic cationic (at physiological pH) and hydrophobic amino acids present in a selected sequence.

Another protein that is of potential relevant interest in the elucidation of the molecular basis of the interaction of proteins/peptides with the lipid A moiety of LPS is tubulin, the globular protein responsible for microtubule formation in the cytoskeleton. This somewhat ubiquitous protein has been also reported to bind LPS (27). In the primary structure of tubulin, the algorithm predicts seven amino acid sequences fulfilling all the requirements (25), specifically at residues 156–166, 287–299, 335–345, 362–373, 381–390, 383–401, and 408–415. The implications deriving from the interaction of tubulin with LPS may be of particular relevance, especially if one considers the capability of LPS to target various tissues in mammalian hosts and be responsible for the failure of key organs like the liver.

Another category of natural polypeptides that would be of potential interest as a source of molecular information useful for generating novel new synthetic peptides specific for the lipid A moiety of LPS are opioid-related peptides. In fact, lipid A is a pyrogenic molecule with reported somnogenic activity (28), and LPS is known for its ability to induce, through lipid A, an increase in the permeability of the blood-brain barrier

Table 2 Synergism of Synthetic Peptides Designed De Novo with Rifampin and Erythromycin Against *E. coli* IH3080 Evaluated by Minimal Inhibitory Concentration (MIC)

	MIC (μg/ml) at indicated peptide concentration (μg/ml)											
	Rifampin						Erythromycin					
Peptide	0.3	1	3	10	30	100	0.3	1	3	10	30	100
None	10						30					
Deacylpolymyxin B	10	0.1	0.01	—[a]	—	—	10	3	1	—	—	—
KFFKFFKFF	10	1	0.03	0.03	0.01	—	30	30	1	3	1	—
IKFLKFLKFL	10	0.3	0.01	0.01	—	—	30	10	1	1	—	—
CKFKFKFKFC [S — S]	10	10	3	1	0.1	—	30	30	30	30	1	—
IKTKCKFLKKC [S — S]	10	10	10	3	3	1	30	30	30	30	30	30
IRTRCRFLRRC [S — S]	3	3	3	1	1	0.1	30	30	30	10	1	1

[a]The peptide alone inhibited growth.

(29) and the release of proinflammatory cytokines in brain tissue (30) and to reproduce effects mimicking those of opioid peptides (31).

Inflammation is a process involving several mediators, and it is now recognized that LPS-induced inflammation in the brain of mice, following an intravenous challenge, results in the upregulation of neurotactin, a newly discovered membrane-anchored small protein of the chemokine families, shortly after LPS challenge (32). With this in mind, it could be important to note that the sensation of pain follows the activation of nociceptors, primary sensory neurons located on cells, which are targets for opioids and are specialized to detect tissue damage in the periphery and in the central nervous system (33). Nociceptin or Orphanin FQ is a new heptadecapeptide discovered in brain tissue of mammals, which resembles the opioid peptide dynorphin A and acts as an endogenous agonist of the opioid receptor-like 1 (ORL1), a new G-protein coupled receptor (34,35). ORL1 is an orphan receptor, resembling opioid receptors, whose cDNA has been detected in brain tissue of both humans and mice. Nociceptin increases reactivity to pain by stimulating the ORL1 receptor, a process that can therefore be defined as no-

Table 3 Synergism of Synthetic Peptides Designed De Novo with Rifampin Against *Klebsiella pneumoniae* and *E. cloacae* Evaluated by Minimal Inhibitory Concentration (MIC)

	MIC (μg/ml) of rifampin at indicated peptide concentration (μg/ml)							
	K. pneumoniae KY12854				*E. cloacae* KY12645			
Peptide	3	10	30	100	3	10	30	100
None	10				10			
Deacylpolymyxin B	0.3	0.1	NT	NT	1	1	NT	NT
KFFKFFKFF	0.3	0.1	0.03	0.01	0.1	0.1	0.03	0.01
IKFLKFLKFL	0.3	0.03	0.01	—[a]	0.03	0.03	0.01	0.01
CKFKFKFKFC [S — S]	10	10	1	0.3	10	3	1	0.3
IKTKCKFLKKC [S — S]	10	10	10	10	10	10	10	3
IRTRCRFLRRC [S — S]	10	10	3	1	10	10	1	1

NT, Not tested.
[a]The peptide alone inhibited growth.

ciceptive stimulation of a nociceptor. Of considerable potential interest is the fact that nociceptin appears to be unique within the family of dynorphin-related peptides, which fulfills all the criteria required by the algorithm for a peptide sequence efficiently binding lipid A and potentially resulting in its detoxification. This neurologic peptide has therefore been evaluated in a variety of in vitro and in vivo assays for its ability to neutralize the biological effects of LPS and has shown a remarkable ability to bind and neutralize LPS in all assays performed (25). Thus, there is a strong suggestion that the opioid-related peptide nociceptin might to date well serve as a potential target of LPS in the central and peripheral nervous system of mammalians. It is our hypothesis that it might function as an important recognition system, serving to alert the host's defenses on the basis of an imbalance in the nociceptin/nociceptor system. This reciprocal imbalance could in turn serve in mammals as a physiological detector triggering the early biological effects of an LPS insult (e.g., inflammation, fever) that follow gram-negative bacterial infections.

DETOXIFICATION MECHANISM(S) OF SAEP

The biological activity of SAEP and PMXB in different animal models of endotoxin-mediated cytokine release and lethality is dose-dependent in experiments in which R- and S-chemotype LPS has been used for either local or systemic challenge (8,9,13) despite the fact that in in vitro studies the binding of SAEP and PMXB to lipid A is stoichiometric (6,8). This experimental observation is most likely explained by the likely antagonistic effects exerted by SAEP and PMXB on the lipid A moiety of LPS, through an active competition with receptor proteins on cells and tissues of the mammalian host (9). The fact, as discussed earlier, that serum LBP and soluble or cell-associated CD14 contain amino acid sequences with characteristics comparable to SAEP would support this conclusion.

However, other experimental observations merit discussion. For instance, binding of SAEP and PMXB to lipid A in undiluted serum does not occur at the stoichiometry observed in aqueous solutions when measured by two independent methods [e.g., surface plasmon resonance (SPR) and equilibrium molecular dialysis (9)]. The presence of serum therefore, appears to interfere with the efficiency of binding, and a significant dilution of serum is required in order to restore full binding activity to the level observed in aqueous solutions. Since neither ionic strength nor pH of the medium offers reasonable explanations for this discrepancy, as binding to lipid A by SAEP and PMXB is not affected by these two parameters in a broad range of values (8), the observed interference may be due to either the presence of LPS-binding components or intrinsic physical-chemical features of the serum environment. Despite these in vitro observations, SAEP are very active in the neutralization of the toxic effects associated with LPS when given prophylactically and therapeutically in vivo (8,9,13,17). These effects are manifest even within a time frame of treatment broader than the experimentally observed half-life time for SAEP in the bloodstream (9). For instance, LPS-dependent production of tumor necrosis factor (TNF), the proinflammatory cytokine generally recognized as responsible for many of the toxic effects induced by LPS, is significantly inhibited by SAEP in the organs, tissues, and sera of animals challenged by LPS, either intravenously, intraperitoneally, or intradermally (9). On these bases, it is reasonable to postulate that the biological manifestations of SAEP activity as an LPS neutralization event occur at the tissue level rather than in the serum.

There are, however, recent findings concerning novel properties of cationic amphipathic peptides that suggest an additional molecular mechanism leading to the inhibition of LPS-induced effects. It has been reported that, at least in vitro, PMXB can serve as a selective and potent antagonist of calmodulin through the formation of a stable molecular complex (36). Also, D-amino-acid analogs of the peptide antibiotic melittin bind to calmodulin, which is remarkably tolerant sterically, and the resulting complexes have been shown to be highly stabilized by van der Waals forces (37). Since calmodulin is a multifunctional protein often involved in the cellular signaling pathways of mammalian cells, where it plays the role of a phosphokinase activator, the capability of cationic amphipathic peptides to interact with calmodulin could result in the inhibition of the LPS-induced signal pathway by a pathway independent of its ability to interact with LPS. This hypothesis would be supported by the observation that calmodulin is a well-characterized subunit of the inducible nitric oxide synthase (NOS) from macrophages (38) and human hepatocytes (39), the enzyme responsible for the synthesis of the biological mediator nitric oxide, a potent hypotensive agent, which can be overproduced in the vasculature after exposure to LPS (40). In this respect, our laboratory has investigated the capability of SAEP to inhibit in vitro the enhancing effect of calmodulin on the phosphodiesterase activity, which leads to the degradation of the cyclic-AMP to 5′-AMP.

tive only of lipid A–specific sequences present in the primary structure of natural polypeptides, the variety of peptide structures evaluated in various studies suggest that the physical-chemical characteristics of SAEP might also represent the primary target for lipid A in the conformationally defined structure of proteins. Also, as in the case of the opioid-like peptide nociceptin, the developed algorithm might help the discovery of novel LPS-binding polypeptides whose binding activity for LPS is still unknown or not yet related to their biological function in nature. The size of peptide sequences optimally determined to bind to lipid A in aqueous solvents (8,13) is comparable to the size reported for epitopes recognized by monoclonal antibodies (45,46) as well as for interaction with the major histocompatibility complex class I molecules (47). This consideration would allow the interesting speculation that the mechanism of LPS recognition by natural polypeptides may well bear similarities to the immunorecognition system. Finally, several different clinical uses of anti-LPS synthetic peptides can be envisioned that, if successful in the near future, would allow innovative approaches in clinical medicine for prophylaxis and treatment of diseases caused by gram-negative bacteria and the related effects due to the release of LPS during the infectious process, such as endotoxic shock.

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