Lipid A, the conserved portion of endotoxin or lipopolysaccharide, is the major mediator of septic shock, and therefore endotoxin-neutralizing molecules could have important clinical applications. The crystal structure of recombinant Limulus anti-lipopolysaccharide factor (rLALF) (Hoess, A., Watson, S., Siber, G. R., and Liddington, R. (1993) EMBO J. 12, 3351–3356), has been used to design synthetic peptides comprising different parts of the exposed amphipathic loop in the proposed endotoxin-binding domain of rLALF. We investigated the minimal requirements of rLALF for endotoxin and lipid A binding with linear 10-mer peptides. Only one linear peptide, corresponding to amino acids 36–45 of rLALF, was able to bind lipid A and endotoxin above background levels. Cyclic peptides, however, bind lipid A and endotoxin with high affinity, presumably by mimicking the three-dimensional characteristics of the exposed hairpin loop. The cyclic peptide including amino acids 36–47, LALF-14, has a lipid A binding activity comparable to the high affinity endotoxin-binding peptide polymyxin B. LALF-14 has an improved serum half-life compared with its linear counterpart, and it is not toxic for cultured human monocytes or red blood cells. In mice, it blocks tumor necrosis factor-alpha induction after endotoxin challenge. The characterization of the minimal endotoxin-binding domain of rLALF and, importantly, its structure provided a basis for designing small molecules that could have prophylactic and/or therapeutic properties in humans for the management of septic shock.

In humans, septic shock and the severe pathological changes associated with it is responsible for about 100,000 deaths in the United States annually (2), and no specific drugs are as yet available. Septic shock arises from a cascade of molecular and cellular events following infection by micro-organisms, predominantly Gram-negative bacteria (3). The onset of shock is due to the interaction of bacterial endotoxins (lipopolysaccharide; LPS)\(^1\) with membrane-bound receptors on macrophages and blood monocytes (4) or various serum proteins (5–7). Lipid A, the conserved region of LPS, has been identified as the toxic component of LPS (8, 9) and hence represents an ideal target for drug development.

Exposure of hemocytes of horseshoe crabs to LPS results in the activation of an intracellular coagulation cascade (10), a defense against microbial invasion (11). The system consists of several proteins, including one that inhibits the cascade, called anti-LPS factor (12). Limulus anti-LPS factor (LALF) is a small (101 amino acids) basic protein (13, 14), which binds and neutralizes LPS (15) and has a strong antibacterial effect on the growth of Gram-negative R-type bacteria (12).

Although there are numerous biomolecules that bind the lipid A portion, the number of substances that effectively neutralize it in vivo is few. Natural and recombinant Limulus anti-LPS factor (rLALF) have been tested on various mammalian target cells and in animal models of septic shock. Both forms inhibit endotoxin-mediated activation of cultured endothelial cells (16) and B cells (17). In animals, rLALF reduces mortality when administered before or after LPS challenge (17, 18) or Gram-negative bacterial infection (19). But antigenicity and toxic side effects of rLALF, as observed in animal experiments, could render it unsuitable for human therapy.

Recent approaches to develop molecules that neutralize endotoxin have concentrated on characterizing lipid A-binding regions from LPS-binding peptides and proteins. This includes synthetic peptides derived from sequences of polymyxin B (20), Tachypleus anti-LPS factor (TALF) (21), rLALF (22), bactericidal/permeability-increasing protein (BPI) (23), CAP-18 (24, 25), and lipopolysaccharide-binding protein (LBP) (26).

The only crystal structure of an endotoxin-neutralizing protein, rLALF, provided a detailed view of a potential LPS-binding site (1). This domain of rLALF is included between amino acids 31 and 52 and is distinguished by an alternating series of positively charged and hydrophobic residues that, by virtue of the extended beta-conformation, point in opposite directions and form a positively charged amphipathic loop. A synthetic peptide comprising the entire loop sequence of rLALF was previously found to bind specifically to LPS (22). Such information provides an excellent basis for designing potentially useful therapeutic molecules. For example, the importance of the amphipathic loop structure can be investigated by introducing

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\(^{1}\) The abbreviations used are: LPS, lipopolysaccharide; LALF, Limulus anti-LPS factor; rLALF, recombinant Limulus anti-LPS factor; LBP, lipopolysaccharide-binding protein; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PBST, PBS-Tween buffer; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FCS, fetal calf serum; LAL, Limulus amebocyte lysate.
appropriate disulfide bonds to create a cyclic conformation. However, previous investigations with cysteine- and non-cysteine-containing peptides derived from TALF, a protein highly homologous with LALF, showed little difference in LPS binding activity (21).

This paper describes the investigation of the 22 amino acid long, positively charged, amphipathic LPS-binding domain with a panel of synthetic peptides. Our results show that only cyclic peptides, which are structural mimics of a distinct part of the LALF-loop described above, and hence might maintain the authentic conformation, bind lipid A and LPS with high affinity and effectively neutralize LPS. This demonstrates the importance of three-dimensional structural analyses in the design of small molecules that could have prophylactic and/or therapeutic properties in humans for the prevention of septic shock.

MATERIALS AND METHODS

Peptide Synthesis—Peptides, were prepared on an automated peptide synthesizer (ABIMED) using a standard Fmoc (N-(9-fluorenylethoxycarbonyl) machine protocol. For detection purposes, all peptides were biotinylated using 2 eq of biotin activated with 2 eq of benzotriazole-1-yl-oxy-tripyrrolidino-phosphonium hexafluorophosphate and 4 eq of N-methylmorpholine. The cleaved peptides were worked up according to the manufacturer’s protocols and purified with reversed-phase HPLC on C-18 columns above 95%. Cyclic peptides were obtained by incubating cysteine-containing peptides in 10% Me2SO for 24 h at room temperature (27). The peptides were then purified by reversed-phase HPLC to purities above 95%. The characterization of the peptides was done by laser desorption-time of flight (LD-TOF) mass spectroscopy.

Inhibition of LPS-induced TNF-α Release in Mice—10 µg of LPS (Salmonella enteritidis), premixed with 100 µg of peptide in 100 µl of PBS, or 100 µg of polymyxin B in 100 µl of PBS, was injected intraperitoneally into BALB/c mice (5/group). 10 µg of LPS in 100 µl of PBS injected intraperitoneally was used as positive control. Animals were bled after 60 min and blood was stored for subsequent TNF-α assays at −80 °C. The TNF-α ELISA was performed according to the manufacturer’s instructions (Genzyme) using 50-µl blood samples. The amounts of TNF-α were quantified by using purified recombinant TNF-α as standard (Genzyme).

RESULTS

Design of Linear Peptides—The region of LALF responsible for LPS binding is located between amino acids 32 and 51 (Fig. 1) and is formed by two strands (assigned strands 2 and 3 in the structural model of rLALF), which at the top form a hairpin loop and at the bottom are connected by a disulfide bridge (1, 22). To further investigate the minimal domain required for LPS binding and neutralization, we synthesized four overlapping linear peptides, each 10 amino acids in length and comprising parts of this domain (Table I). LL:32–41 and LL:42–51 were biotinylated using 2 eq of biotin activated with 2 eq of benzotriazole-1-yl-oxy-tripyrrolidino-phosphonium hexafluorophosphate and 4 eq of N-methylmorpholine. The cleaved peptides were worked up according to the manufacturer’s protocols and purified with reversed-phase HPLC on C-18 columns above 95%. Cyclic peptides were obtained by incubating cysteine-containing peptides in 10% Me2SO for 24 h at room temperature (27). The peptides were then purified by reversed-phase HPLC to purities above 95%. The characterization of the peptides was done by laser desorption-time of flight (LD-TOF) mass spectroscopy.

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Minimal Length of the LPS-binding Domain of LALF—To evaluate binding activities of the peptides to lipid A, we performed ELISA assays in which lipid A was coated on a microtiter plate and further incubated with increasing amounts of biotin-labeled peptides (Fig. 2A). All linear peptides derived from TALF were able to bind lipid A. NEU-10 had binding activities higher than the uncharged peptide NEU-10. The strongest LPS binding activity was displayed by peptide LL:36–45, while the other peptides bound lipid A with activities comparable to the
High Affinity Endotoxin-binding and Neutralizing Peptides

Design of Synthetic Peptides—In the crystal structure, the LPS-binding domain of rLALF is presented in a loop conformation (Fig. 1), which is stabilized by a disulfide bond at the stem of the loop and the H-bond interactions of the β-sheet structure. As most of these features are missing in the linear peptide LL:36–45, it is unlikely that the linear peptide LL:36–45 adopts a loop similar to that seen in the crystal structure. We therefore investigated the importance of the cyclic conformation for high affinity LPS binding. We synthesized several cyclic peptides having cysteines at either end of the molecule (Fig. 1, Table I). LALF-10, looping the entire loop sequence of rLALF, was previously found to bind specifically to LPS (22) and served as a positive control in the LPS-binding assays. LALF-10, LALF-11, and LALF-14 comprised 8, 9, and 12 amino acids, respectively, of the exposed hairpin loop. LALF-10 and LALF-11 represented different lengths of the most active linear LPS-binding peptide LL:36–45, while LALF-14 had two additional amino acids at the C terminus and consisted of residues 36–47. RETL-10 comprised the same amino acids as LALF-10, but synthesized in the reversed orientation. The design of the cyclic peptides was planned in such a way that neighboring residues in the β-sheet of the rLALF-loop structure were replaced by cysteines to obtain a cyclic conformation of the peptides identical to the orientation seen in the rLALF crystal structure (Fig. 1). LALF-10 and LALF-11 had proline 37 and tyrosine 46, and LALF-14 had isoleucine 35 and glycine 48, changed to cysteines.

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Structural Requirements for LPS Binding—We compared the lipid A binding activities of the linear peptide LL:36–45 with the cyclic peptides in an ELISA with solid phase-adsorbed lipid A (Fig. 2B). PA-10 and NEU-10 were used as control peptides as in Fig. 2A. LALF-10, LALF-11, LALF-14, and LALF-22 bound lipid A with considerably higher activity than the linear peptide LL:36–45, indicating that a cyclic conformation is necessary for high affinity binding. LALF-14 was able to bind lipid A with an activity slightly better than LALF-10 and LALF-11 and almost identical to that of LALF-22. The peptide synthesized in the reversed orientation of LALF-10, RETL-10, showed little lipid A binding activity, confirming that the precise structural arrangement of residues in the cyclic peptide was important for high affinity LPS binding. This result indicated that the minimal sequence for LPS binding resides within amino acid residues 38–45 (LALF-10). But, as the N-terminal free amine group of LALF-10 was not blocked after synthesis, it is possible that the free N terminus of LALF-10, although structurally different, functionally replaces the side chain amino group of lysine in LALF-11. Hence the minimal LPS-binding domain of rLALF should probably be assigned to residues 36–45 (LALF-11).

LPS from different bacteria consists of lipid A attached to polysaccharides of different lengths. To see whether the saccharide moiety had an influence on the lipid A binding of the peptide, we investigated binding of a cyclic peptide to different kinds of LPS (Fig. 2C). LALF-14 was added to an ELISA-plate coated with lipid A or LPS. The forms of lipopolysaccharide clearly above background level, but with slightly different affinities. S. enteritidis LPS and lipid A were bound with the highest affinities.

Specificity and Affinity of Lipid A-binding Peptides—To define the specificity of the peptides in binding lipid A, competition experiments were performed using rLALF and serum. The binding affinities of the peptides were compared with a high affinity LPS binder, polymyxin B. This is a positively charged amphipathic cyclic oligopeptide linked to a single fatty acid (28), resembling a similar molecular weight as the synthetic peptides. Polymyxin B has been shown to bind lipid A with a dissociation constant of approximately 10⁻⁷ M (29) and to inhibit rLALF lipid A binding (22). Lipid A was adsorbed on a

![Image](101x471 to 245x729)

**Fig. 1.** Schematic drawing of the loop structure of rLALF. The directions of the amino acid (single-letter code) side chains are indicated. Solid bonds indicate side chains pointing out of the plane of the diagram; dashed bonds are into the plane. Cysteines were introduced pairwise instead of the authentic amino acid residues at positions indicated with a dotted line representing the resulting disulfide bridge.

**Table I**

| Peptide | Sequence     |
|---------|--------------|
| LL:32–41: | HYRIKPTFRR |
| LL:36–45: | KPTFRLKWK |
| LL:42–51: | LKWKYKGKFW |
| LALF-10 (38–45): | G-C- TFRRLKW-C-G |
| LALF-11 (36/38–45): | K-C- TFRRLKW-C-G |
| LALF-14 (36–47): | G-C- KPTFRLKWKY-C-G |
| LALF-22 (31–52): | G-C- HYRIKPTFRLKWKYKGKFW-C-G |
| LL:45–36: | KWKLRRTPK |
| RETL-10 (45–38): | G-C- KWLKRFT-C-G |
| PA-10: | PRWTFKLLK |
| NEU-10: | GAPQQDLNNTMK |

Table I: Design of synthetic peptides: sequence of peptide analogs of rLALF

The amino acid sequence of rLALF comprising residues 30–53 with secondary structure elements indicated (s = strand) (1) is shown at top. Below are the sequences of the synthetic peptides comprising different parts of the potential LPS-binding site from rLALF (underlined). All peptides containing 2 cysteines were oxidized to obtain a cyclic conformation (LALF-10, LALF-11, LALF-14, LALF-22, and RETL-10). The numbers in the brackets of the cyclic peptides and the numbers in the linear peptide designation (LL) indicate the amino acid residues in the authentic protein. The two control peptides used were PA-10, which resembles the scrambled version of the peptide comprising amino acid residues 36–45, and NEU-10, which has a neutral net charge.
microtiter plate and incubated with a mixture of rLALF and increasing amounts of peptide (Fig. 3A). If the peptides bind specifically to the same epitope on lipid A as rLALF, they should compete with rLALF for lipid A binding. Polymyxin B was used as a positive control. At 10 μg/ml, polymyxin B inhibited 80% of the rLALF/lipid A binding. The control peptides NEU-10 and PA-10 were not able to significantly compete with rLALF, confirming that positive charges and amphipathicity alone were not sufficient for high affinity LPS binding. Surprisingly, the linear peptide LL:36–45 only weakly inhibited rLALF/lipid A binding (15%), while all cyclic peptides had a strong effect on the binding of rLALF to lipid A (between 75% and 85% inhibition), demonstrating their high binding specificity for lipid A. Furthermore, the cyclic peptides competed with rLALF for lipid A binding as efficiently as did polymyxin B, indicating a similar LPS/lipid A-binding affinity as polymyxin B.

To investigate if biotin labeling influenced the competition behavior of the peptides, several labeled and unlabeled peptides were compared in similar assays. No differences in the inhibition of the LALF/lipid A binding were observed for LL:36–45, LALF-10, LALF-22, and NEU-10 (data not shown), implying that biotin labeling does not affect binding ability of the peptides.

Serum contains many LPS-binding components, including LBP and other LPS-binding proteins (5). Therefore, a peptide displaying high LPS-binding affinity in the presence of serum should be the preferred candidate for any in vivo applications. To screen potent LPS-binding peptides, which could be used for an in vivo application to block LPS toxicity, we developed an assay that includes serum instead of buffer. We measured the binding of labeled peptides and rLALF to lipid A at a serum concentration of 10% in RPMI 1640 and compared it to their binding in RPMI 1640 only. For rLALF, a serum concentration-dependent decrease in its lipid A binding is observed (Fig. 3C).

For LALF-10, LALF-11 and LALF-14, the lipid A binding activity also decreased, but the peptides were clearly able to bind lipid A in 10% serum (Fig. 3B). To further investigate if the binding specificity of the LALF-derived peptides to lipid A is influenced by serum, we performed competition experiments with the rLALF/lipid A binding in which buffer (PBST) is replaced by medium and serum. This assay again allows us to compare the peptide lipid A binding activity with that of polymyxin B. We tested the inhibition of the rLALF/lipid A binding in medium only and medium plus serum (Fig. 3C). The strong inhibition in buffer of the rLALF/lipid A binding by LALF-derived peptides was also observed in the presence of serum. However, in all cases a small decrease (~10–20%) of the competition capacity of the peptides in the presence of serum was observed. Again, the affinity of cyclic LALF-derived peptides in serum is comparable to that of polymyxin B.

Biological Behavior of Lipid A-binding Peptides—An important feature in developing peptides for an in vivo application to treat Gram-negative bacterial septic shock is not only effective and specific binding of LPS, but also their behavior in biological

![Fig. 2. Determination of the minimal length and structural requirements of the LPS-binding domain of rLALF.](image_url)
FIG. 3. Specificity and affinity of lipid A-binding peptides. A, competition of rLALF/lipid A binding by rLALF-derived peptides. 10.0 μg/ml peptide mixed with 0.25 μg/ml rLALF was incubated with lipid A (0.5 μg/ml), which was adsorbed to a microtiter plate. The ELISA was developed with antiserum against rLALF and an alkaline phosphatase-conjugated second antibody. The deviation between the samples was less than 8.7%. B, influence of serum on the lipid A binding activity of the biotinylated peptides. As binding buffers, PBST, medium only (medium) or medium with 10% serum (medium/10%FCS) was used. The assay was performed as in Fig. 2A. C, competition of rLALF/lipid A binding by rLALF-derived peptides in buffer (PBST), in the presence of medium (medium) and medium containing 10% FCS (medium/10%FCS). 10.0 μg/ml peptide mixed with 0.25 μg/ml rLALF was incubated with lipid A (0.5 μg/ml), which was adsorbed to a microtiter plate. The assay was performed as in Fig. 3A. The deviation between the samples was less than 10.3%.
systems. Problems that often arise in animal experiments are due to toxicity, as in the case with polymyxin B, or a very short half-life in the circulating system. To assess these features, we investigated some of the synthetic peptides for their hemolytic activity and their ability to permeabilize cultured cells (Table II). The LALF-derived peptides LL:36–45, LALF-10, and LALF-14 had little hemolytic activity. The toxicity of the peptides for mammalian cells was tested by incubation with human monocytes and analyzed using MTT, a substance that could only be metabolized by healthy cells. The peptides had only a modest effect on cell permeabilization. Compared with polymyxin B, known to be non-hemolytic, LL:35–46, LALF-10, and LALF-14 showed a slightly increased hemolytic activity, and, in contrast to polymyxin B, the LALF-derived peptides had a 5–10-fold reduced lytic activity for cultured monocytes.

The stability of the peptides was tested by investigating their half-lives in serum at 37°C. Peptides were incubated in fetal calf serum, and their stability was monitored by HPLC-analysis. The linear peptide LL:36–45 showed a half-life of 17 min, while the cyclic peptides LALF-11 and LALF-14 showed half-lives of 77 and 84 min, respectively. This demonstrated that a cyclic conformation of the peptides is not only important for high affinity LPS binding, but in addition has the advantage of conferring greater stability in plasma.

LPS Neutralization in Vitro—To assess the neutralization capacity of the synthetic peptides, we performed two different assays, which were utilized to test LPS toxicity. LPS neutralization in vitro was tested by the chromogenic Limulus amebocyte lysate assay (30), allowing detection of free LPS in pg/ml amounts. The most potent inhibitors of the LALF gelling reaction were rLALF and polymyxin B, showing almost 100% inhibition (Fig. 4A). The peptide concentration used in this assay is comparable to polymyxin B and 100-fold higher than rLALF. The linear peptide LL:35–46 as well as PA-10 inhibited gelling only weakly (~20%), while NEU-10 was not able to block the reaction. The cyclic peptides LALF-10, LALF-11, LALF-14, and LALF-22 were able to inhibit gelling between 60% and 80%, confirming that a cyclic conformation is important not only for LPS binding but also for LPS neutralization.

Inhibition of LPS-induced TNF-α Release in Mice—The toxicity of LPS in animals and humans is associated with an increase of cytokines, such as TNF-α. In mice the maximum levels of TNF-α were after 1–2 h after LPS-exposure. The key mediators of this response have been shown to be LBP and CD14, both lipid A-binding proteins (7, 31). rLALF has been shown to effectively inhibit LPS-induced TNF-α expression (32), presumably by blocking the lipid A binding of LBP or CD14. Whether the peptides possess a similar activity was tested by assaying the ability of LALF-14 to block the TNF-α response after LPS challenge in mice (Fig. 4B). Groups of five mice were co-injected with LPS plus buffer, LALF-14, or polymyxin B. For comparison, LALF-14 was used at the same molar concentration as polymyxin B. Polymyxin B was able to inhibit TNF-α induction by 90%. LALF-14 was similar to polymyxin B a potent inhibitor of the TNF-α overproduction, reducing the amount of TNF-α by 80% compared to the control.

DISCUSSION

Therapeutic strategies for the treatment of septic shock in humans have focused on the neutralization of LPS and its endogenous mediators (33–35), but no specific drugs are yet available. LPS is considered to be the primary stimulus for the entire cascade of endogenous mediators. Agents directed against LPS would therefore have the advantage over agents directed against the endogenous mediators by preventing the action of not only individual members of the pro-inflammatory cascade but all of them. Furthermore, since early diagnosis of septic shock, which is very difficult, is considered to be another crucial parameter, prophylaxis could be a valuable approach to disease management. Thus, a demand exists for molecules that would have LPS neutralizing activity.

LALF, previously identified as a potent anti-coagulant found in Limulus hemocytes (13), has been shown to bind and neutralize LPS (15). In our report, we have investigated the LPS-binding region of LALF in detail and characterized the minimal binding domain spanning 8–10 amino acids located between residues 36–45. Originally, it was suggested (14) that amino acids 40–51 of LALF, comprising strand 3 in the crystal structure (1), are important for LPS binding. Recently, the LPS-binding domain of Tachypleus anti-LPS factor (TALF), was assigned to this region (21). Our linear peptide homologous to this region, LL:42–51, clearly showed less binding activity than LL:36–45, a peptide region not investigated in TALF (21).

Importantly, we have shown that high affinity LPS binding only was achieved after cyclization, leading to peptides with an affinity comparable to polymyxin B ($K_D = 10^{-7}$ M). Compared to the LPS-binding affinity of the holoprotein rLALF (17), this represents approximately a 10-fold loss in binding affinity of the peptides. Decreased binding affinity was reported in other cases when binding constants of peptides representing domains of proteins were compared with those of the holoprotein, in particular for the LPS-binding proteins BPI (23) and LBP (26). This is in accordance with our results and supports the identification of the LPS-binding domain being located between amino acid residues 36 and 45.

Analyses of the LPS binding activity of polymyxin B-derived peptides has shown that a cyclic conformation was a crucial determinant for high affinity LPS binding (20). A 10-amino acid residue, cyclic peptide derived from polymyxin B showed higher binding activity to lipid A compared to its linear counterpart. However, this result was not observed with longer peptides derived from LALF, such as LALF-22. Oxidized and reduced LALF-22 bound LPS with activities almost equal to those of LPS (data not shown). LALF-22 resembles the entire loop sequence of LALF. Therefore, interactions between the two β-stands could lead to a cyclic loop conformation of LALF-22. A similar finding was reported with peptides 24 residues long, which were derived from TALF (21). Only a small difference in the binding activities between the cysteine-containing and non-cysteine-containing peptides was observed, showing that here too, the disulfide bond was not absolutely essential.

A method used to characterize molecular recognition domains of LPS-binding proteins is the use of peptides scanning the entire protein sequence. In the cases of LBP (26), BPI (23),
and TALF (21), which is highly homologous to LALF, this led to the identification of linear peptides with LPS binding and neutralizing activity. The domains identified therein are overlapping with our predicted regions (1). However, the crystal structure analysis of rLALF provided the knowledge to obtain high affinity peptides derived from rLALF, as we show here that the cyclic conformation of peptides is important for high affinity binding. For LBP and BPI, crystal structures are not yet available, but on the basis of our results obtained with the LALF peptides, we predict that also in these cases cyclization could result in higher affinity LPS-binding peptides.

The cyclic peptides LALF-10, LALF-11, and LALF-14, characterized in this report, fulfill important requirements for use as therapeutics to prevent or treat Gram-negative bacterial sepsis, such as specific and high affinity binding to different kinds of LPS, low toxicity for erythrocytes and cultured eukary-

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**Fig. 4.** LPS neutralization in vitro and in vivo. A, inhibition of LPS-induced gelling of chromogenic *Limulus* amebocyte lysate by synthetic rLALF-derived peptides or by control peptides (2.5 μg/ml) using 100 pg/ml LPS. rLALF (0.25 μg/ml) and polymyxin B (2.5 μg/ml) were used as positive controls. The assay was performed as described under “Experimental Procedures.” B, inhibition of LPS-induced TNF-α release in mice by LALF-14. 10 μg of LPS, alone or premixed with 100 μg of LALF-14 or 100 μg of polymyxin B was injected intraperitoneally into mice (5/group). After 1 h, mice were sacrificed, and collected serum was assayed for TNF-α content by means of an anti-TNF-α ELISA as described under “Experimental Procedures.” The standard deviations between the samples were, for the control, 43.7%, for LALF-14/LPS, 29.8%, and for polymyxin B (PolB)/LPS, 26.6%.
otic cells, and neutralizing activity in vitro and in vivo. The cyclic design of the peptides compared with the linear ones resulted in a longer serum half-life, which has been shown in other cases to be a crucial determinant in successful drug development (36–38). Furthermore, the small size of the peptide could provide a lead compound for the synthesis of peptidomimetics with improved characteristics, yielding molecules that could be used prophylactically to prevent septic shock.

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REFERENCES

1. Hoess, A., Watson, S., Siber, G. R., and Liddington, R. (1993) EMBO J. 12, 3351–3356
2. Parrillo, J. E. (1990) Ann. Intern. Med. 113, 227–242
3. Glaser, M. P., Zanetti, G., Baumgartner, J.-D., and Cohen, J. (1991) Lancet 338, 732–736
4. Morrison, D. C., Lei, M. G., Kirikae, T., and Chen, T. Y. (1993) J. Biol. Chem. 268, 212–226
5. Wright, S. D., Ramos, R. A., Patel, M., and Miller, D. S. (1992) J. Exp. Med. 176, 719–727
6. Schumann, R., Leong, S. R., Flaggs, G. W., Gray, P. W., Wright, S. D., Mathison, J. C., Tobias, P. S., and Ulevitch R. J. (1990) Science 249, 1429–1431.
7. Tobias, P. S., and Ulevitch, R. J. (1993) Immunobiol. 187, 227–232
8. Eitschel, E. T., and Brade, H. (1992) Sci. Am. 267, 54–61
9. Retschel, E., Kirikae, T., Schade, U., Mamat, U., Schmidt, G., Loppmow, H., Ulmer, A., Zahringer, U., Seyerl, U., DiPadova, F., Schreier, M., and Brade, H. (1994) FEBS J. 302, 217–225
10. Iwanaga, S., Morita, T., Miyata, T., Nakamura, T., and Aketagawa, J. (1986) J. Proc. Chem. 5, 253–268
11. Nachum, R., Watson, S. W., Sullivan, J. D., and Siegel, S. E. (1979) J. Infect. Dis. 139, 290–299
12. Morita, T., Ohtsubo, S., Nakamura, T., Tanaka, S., Iwanaga, I., Ohashi, K., and Niwa, M. (1985) J. Biochem. (Tokyo) 97, 1611–1620
13. Aketagawa, J., Miyata, T., Ohtsubo, S., Nakamura, T., Morita, T., Hayashida, H., Miyata, T., Shionoishi, Y., Takas, T., and Iwanaga, S. (1986) J. Biol. Chem. 261, 7357–7365
14. Muta, T., Miyata, T., Tokunaga, F., Nakamura, T., and Iwanaga, S. (1987) J. Biochem. (Tokyo) 101, 1321–1330
15. Wainwright, N. R., Miller, R. J., Paus, E., Novitsky, T. J., Fletcher, M. A., McKenna, T. M., and Williams, T. (1990) in Cellular and Molecular Aspects of Endotoxin Reactions (Nowotny, A., Spitzer, J. J., and Ziegler, E. J., eds) pp. 315–325, Elsevier Science Publishers B.V., New York
16. Desch, C. O’Hara, P., and Harlan J. (1989) Infect. Immun. 57, 1612–1614
17. Warren, G., Glennon, M. L., Wainwright, N. R., Amato, S. F., Black, K. M., Kirsch, S. J., Riveau, G. R., Whyte, R. I., Zapel, W. M., and Novitsky, T. J. (1992) Infect. Immun. 60, 2596–2513
18. Alpert, G., Baldwin, G., Thompson, C. M., Wainwright, N. R., Novitsky, T. J., Gillis, Z., Parsonnet, J., Fleisher, M. A., and Siber, G. R. (1992) J. Infect. Dis. 165, 494–500
19. Saladinio, R., Garcia, C. T., Thompson, C. M., Hammer, B. K., Parsonnet, J., Novitsky, T. J., Siber, G. R., and Fleisher, G. R. (1994) Circ. Shock 42, 104–110
20. Rustici, A., Velucchi, M., Faggioni, R., Sironi, M., Quartaert, S., Green, B., and Porro, M. (1993) Science 259, 361–365
21. Kloczewiak, M., Black, K. M., Loiselle, P., Cavaillon, J. M., Wainwright, N., and Warren, H. S. (1994) J. Infect. Dis. 170, 1490–1497
22. Hoess, A., Schneider-Mergener, J., and Liddington, R. (1995) Prog. Clin. Biol. Res. 392, 237–337
23. Little, R. G., Kelner, D. N., Lim, E., Burke, D. J., and Conlon, P. J. (1994) J. Biol. Chem. 269, 1865–1872
24. Hirata, M., Shimomura, Y., Yoshida, M., Wright S. C., and Larrick, L. W. (1994) Prog. Clin. Biol. Res. 388, 147–159
25. Larrick, J. W., Hirata, M., Zheng, H., Zhong, J., Bolin, D., Cavaillon, J. M., Warren, H. S., and Wright S. C. (1994) J. Immunol. 153, 231–240
26. Taylor, A. H., Heavner, G., Nedelman, M., Sherris, D., Brant, E., Knight, D., and Ghayreb, J. (1995) J. Biol. Chem. 270, 17934–17938
27. Tam, J. P., Wu, C. R., Liu, W., and Zhang, J. W. (1991) J. Am. Chem. Soc. 113, 6657–6662
28. Morrison, D. C., and Jacobs, D. M. (1976) Immunchemistry 13, 813–818
29. Vaara, M. (1992) Microbiol. Rev. 56, 393–411
30. Levin, J., and Bang, P. B. (1964) Bull. Johns Hopkins Hosp. 115, 265–274
31. Gallay, P., Heumann, D., LeBoy, D., Barras, C., and Luscher, M. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 9935–9938
32. Nelson, D., Kuppermann, N., Fleisher, G. R., Hammer, B. K., Thompson, C. M., Garcia, C. T., Novitsky, T. J., Parsonnet, J., Onderdonk, A., Siber, G. R., and Saladinio, R. (1995) Crit. Care Med. 23, 92–98
33. Alexander, H. L., Doherty, G. M., Buresh, C. M., Venzon, D. J., and Norton, J. A. (1991) J. Exp. Med. 173, 1029
34. Ashkenazi, A., Masters, A. S., Capon, D. J., Chomow, M. S., Figari, I., Ianni, S., Pennica, D., Goeddel, D. V., Palladino, M. A., and Smith, D. H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10535–10541
35. Calandra, T., Baumgartner, J. D., and Glaser, M. P. (1991) Prog. Clin. Biol. Res. 367, 141–149
36. Wong, Y. N., Burcham, D. L., Saxton, P. L., Erickson-Viitanen, S., Grubb, M. F., Quan, C. Y., and Huang, S. M. (1994) Biopharm. Drug. Dispos. 15, 535–44
37. Marastini, M., Salvadori, S., Scaranari, V., Spisani, S., Reali, E., Tranelli, S., and Tomatis, A. (1984) Arzneimittel-Forschung 44, 1073–1076
38. Tschopp, J. F., Driscoll, E. M., Ma, D. Y., Black, S. C., Pieruschka, M. D., and Lucchesi, B. R. (1993) Coron. Artery Dis. 4, 809–817