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A synthetic cyclic peptide derived from *Limulus* anti-lipopolysaccharide factor neutralizes endotoxin *in vitro* and *in vivo*

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KEYWORDS
Septic shock;
Lipopolysaccharide;
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Abstract

Endotoxin, also known as lipopolysaccharide (LPS), is the major mediator of septic shock due to Gram-negative bacterial infections. Recently, much attention has been focused on cationic peptides which possess the potential to detoxify LPS. *Limulus* anti-LPS factor (LALF), a protein found in the horseshoe crab (*Limulus polyphemus*), has been proved with striking anti-LPS effects. We synthesized a cyclic peptide (CLP-19), and then investigated its bioactivity both *in vitro* and *in vivo*. The ability of CLP-19 to neutralize LPS *in vitro* was tested using a *Limulus* amebocyte lysate (LAL) assay and the LPS-binding affinity was measured with an affinity biosensor method. The synthetic peptide $LALF_{31-52}$ (residues 31 to 52 of LALF) was used as the positive control peptide in this study. It was found that CLP-19 exhibited the significant activity to antagonize LPS without observable cytotoxicity effect on mouse macrophages. CLP-19 directly bound to LPS, and neutralized it in a dose-dependent manner in the LAL assay. Moreover, CLP-19 also showed the remarkable ability to protect mice from lethal LPS attack and to inhibit the LPS-induced tumor necrosis factor alpha (TNF-α) release by decreasing serum LPS *in vivo*. Our work suggests that this peptide is worthy of further investigation as a potential anti-LPS agent in the treatment of septic shock.

1. Introduction

Gram-negative bacterial sepsis with resultant multiple organ dysfunction and death continues to be a serious problem in intensive-care patients. Lipopolysaccharide (LPS), also called endotoxin, is considered to play an important role in

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the pathogenesis of gram-negative bacterial sepsis and septic shock [1]. Therapeutic strategies for the treatment of septic shock in humans are currently interested in neutralization of LPS. Compounds that neutralize LPS or its toxic component, the lipid A moiety, may provide a potential source of molecules in the treatment of sepsis [2].

Recently, much attention has been focused on cationic peptides and their derivatives such as polymyxin B [3], bactericidal/permeability increasing protein (BPI) [4], CAP-18 [5], mastoparan peptide [6], and LPS-binding protein (LBP) [7], which possess the potential to neutralize LPS and prevent LPS-triggering cytokines induction in macrophages and even block sepsis in animal models. A small, basic peptide found in hemocytes from both *Tachypleus tridentatus* and *Limulus polyphemus*, the *Limulus* anti-LPS factor (LALF) binds to LPS with high affinity in a dose-dependent manner and accordingly block the bioactivity of LPS [8,9]. It was reported that the region of LALF responsible for LPS binding is located between amino acids 31 and 52 and is characterized by an alternating series of positively charged and hydrophobic residues that form a positively charged amphipathic loop [8]. Vallespi et al. reported that $LALF_{31-52}$ showed preventive and therapeutical effects which obviously increased the survival rate of mice under the LPS attack in a mouse model with peritoneal fulminating sepsis [10,11].

Based on analysis of LALF structural features, we attempted to reconstitute the molecular structure of LALF. CLP-19, a peptide composed of 19 amino acid residues, was synthesized and subsequently treated by amidation and cyclization to improve LPS-binding activity because it was proved that the cyclic conformation and positive charges of the peptides are of great importance for high affinity LPS binding [4]. In this study, the anti-LPS activity of CLP-19 *in vitro* and *in vivo* was evaluated and compared with $LALF_{31-52}$ in order to assess whether our synthetic peptide will be a potential agent in the treatment of septic shock.

2. Materials and methods

2.1. Materials

2.1.1. Animals and cells

Inbred Kunming mice were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The weight of mice on the day of the experiments was 20±2 g. Mouse RAW264.7 cells were obtained from the Department of Pharmacology of the Third Military Medical University (Chongqing, China).

2.1.2. Reagents

LPS from *Escherichia coli O111:B4* was purchased from Sigma (St. Louis, MO, USA). The kinetic turbidimetric LAL kits were from Zhanjiang A&C Biological (Zhanjiang, China). Mouse tumor necrosis factor alpha (TNF-α) ELISA kits were from Jingmei Biotech Company (Beijing, China).

2.1.3. Preparation of peptide CLP-19

CLP-19 was synthesized with the Symphony Peptide Synthesis System (Protein Technologies, Inc., USA), which utilizes Fmoc chemistry via stepwise solid phase peptide assembly starting from a Fmoc-Lys (Boc)-Wang resin. After drying, the resins were cleaved by TFA (trifluoroacetic acid) cocktail solution, and the peptide was purified by HPLC (prepared by Shenzhen Hybio Engineering, Shenzhen, China). No LPS was detected in the peptide solution, as determined by LAL test.

2.2. Methods

2.2.1. Neutralization of endotoxin *in vitro*

The ability of CLP-19 to neutralize LPS *in vitro* was assayed with the LAL test, a sensitive indicator of the presence of free, non-neutralized LPS. Various concentrations of the peptides were incubated with 0.5 EU/ml of LPS at 37 °C for 30 min and LPS alone was the positive control. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent. The kinetic turbidity was measured using a Tube Reader ATi-321 (Lab Kinetics, UK).

2.2.2. Affinity assessment

LPS was immobilized on the surface of a hydrophobic cuvette (Thermo Labsystem, USA), according to the manufacturer's instructions. Briefly, the hydrophobic cuvette was pretreated with 2-propanol and phosphate buffered saline containing 0.025% (w/v) sodium azide and 1 mM EDTA (PBS/AE; pH 7.4). Fifty microliter of 2-propanol and 10 μl of LPS (2 mg/ml in chloroform) were mixed in the cuvette and allowed to bind for 1 min. After the cuvette was washed seven times with 60 μl PBS/AE, the data were collected for 5 min. Then the cuvette was alternately washed with 0.1 M HCl, PBS/AE and 10 mM NaOH seven times. Next, 90 μl BSA (bovine serum albumin, 5 mg/ml) was added, and 5 min later, the cuvette was washed five times with 60 μl PBS/AE. The data were collected after the seventh PBS/AE wash. After LPS was immobilized on the surface of the hydrophobic cuvette, various concentrations of the peptides were added, a binding curve was generated, and the K_d values were measured with the Affinity Sensors IAsys. Additional analyses were performed with the FASTplot and FASTfit software packages (Thermo Labsystem).

2.2.3. Evaluation of LPS-induced TNF-α release from RAW264.7 cells

Mouse RAW264.7 macrophages were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere at 5% CO_2 and 95% air (unless mentioned otherwise). The cell suspensions (1.0×10^6 cells/ml, 0.4 ml) were added into a 96-well plate (Falcon) and incubated at 37 °C for 2 h. Then 20 μl LPS (100 ng/ml) was added followed by 20 μl peptides. Cells incubated with LPS alone and saline

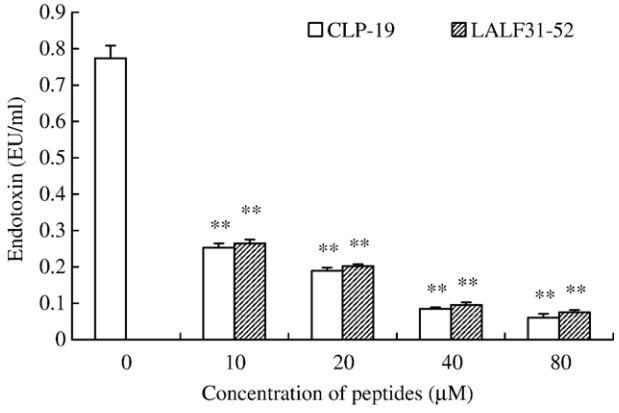

Figure 1 Peptide CLP-19 neutralizes LPS *in vitro*. Various concentrations of CLP-19 and the control peptide $LALF_{31-52}$ were incubated with LPS (0.5 EU/ml) at 37 °C for 30 min. Subsequently, 100 μl of each mixture was added to an equal volume of the LAL reagent. The kinetic turbidity was measured using an ATi-321tube reader. Data are the means±s.d. of triplicate determinations. Other details are as described under Methods. ** $p<0.01$ compared with the LPS alone group. # $p<0.05$, CLP-19 group compared with $LALF_{31-52}$ group.

were used as positive and blank control, respectively. After cells were incubated for 4 h, the levels of TNF-α in the supernatants were analyzed using the ELISA kits.

2.2.4. Cytotoxicity assay

Cytotoxicity was established using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After washing by PBS, RAW264.7 cells were suspended in DMEM supplemented without serum and subsequently plated in a 96-well culture dish (10^4/well). Cells were cultured for 2 h at 37 °C, and then incubated with the peptides (10, 20, 40, and 80 μM) for 4 h. Subsequently, 20 μl of MTT solution (5 mg/ml in PBS) were added in a total volume of 200 μl medium. After incubation for another 4 h, the supernatant was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well. The extinction was measured at 540 nm with a microplate reader.

2.2.5. Experimental mouse endotoxemia model

A model of experimental endotoxemia in Kunming mice was used to characterize the efficacy of our synthetic peptide in protecting an immunological intact host against serious LPS attack. Groups of 10 mice received an intra-peritoneal injection with various doses of peptides (0.025, 0.05, 0.1, 0.2 and 0.4 μmol/kg of body weighs) in 0.1 ml saline or saline alone (0.1 ml), together with LPS. LPS (20 mg/kg of body weigh in 0.1 ml saline), which produced 100% mortality in 24 h, was used to induce experimental endotoxemia. Survival was recorded every 8 h during a period of 168 h.

2.2.6. Endotoxin-neutralization and TNF-α release inhibition in mice serum

For each peptide, mice (n=4) were intraperitoneally injected with peptide (5 mg/kg) or LPS (2 mg/kg) or mixture solution (1:1) of LPS and peptide. Mice given only saline were as blank control. The total injection volume was 0.2 ml per 20 g bodyweight. Sixty minutes after intra-peritoneal injection, blood samples (10 μl) were collected from the sacrificed mice. After dilution (40×by saline), the samples were stored at −80 °C. For LAL test, samples were diluted with saline (10×) and then incubated in 70 °C water for 10 min in order to inactivate heparin. The subsequent procedures were as described above. The levels of TNF-α in the samples were analyzed using the ELISA kits.

2.2.7. Statistics and presentation of data

Each experiment was repeated at least twice and each data point represents the mean of at least three parallel samples. The concentrations are expressed as mean±standard deviation (s.d.). The significance of differences in mouse mortality among groups was analyzed by Chi-squared exact test. The differences in endotoxin and cytokine concentrations and the effects comparison of two peptides were examined by the student's t test. A p value less than

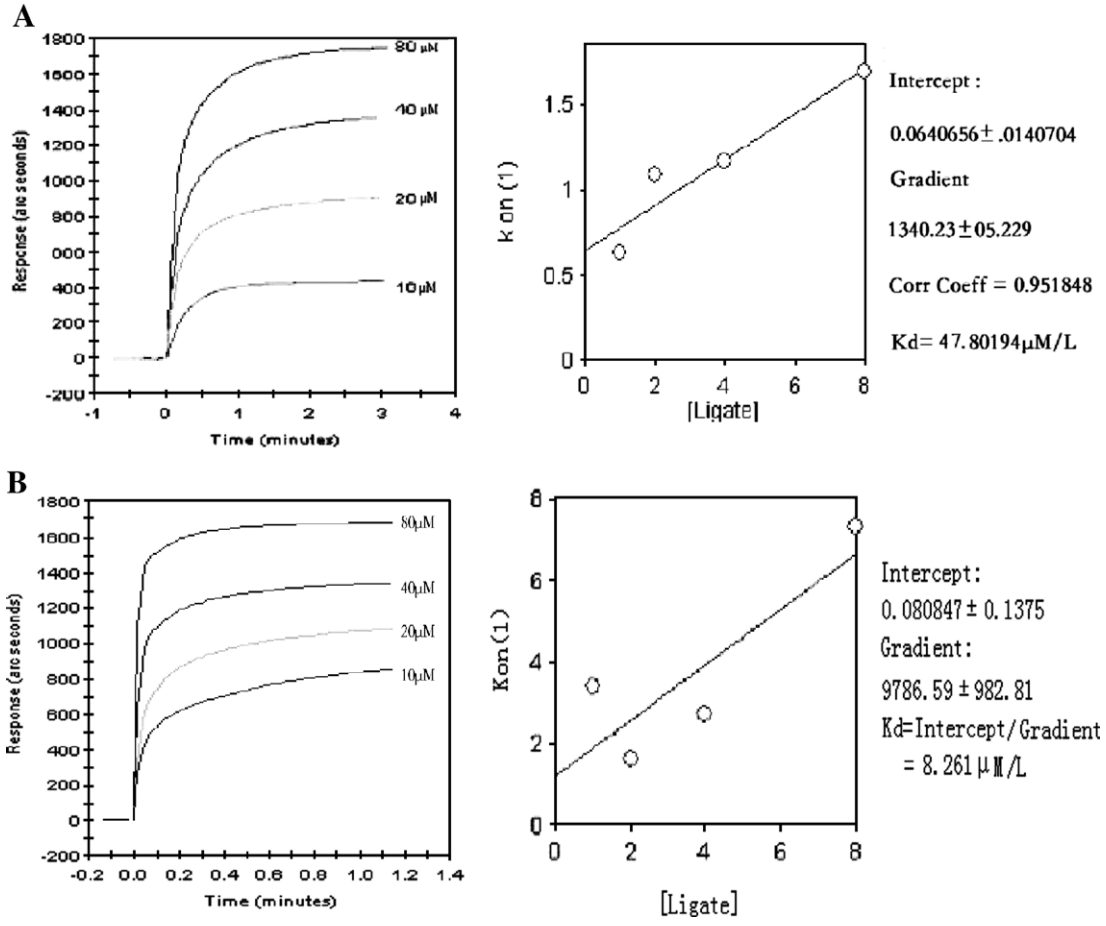

Figure 2 The capacity of peptide binding to LPS. LPS was immobilized on the surface of a hydrophobic cuvette as described in the Materials and methods. Different concentrations of peptides were added to the immobilized cuvette. A binding curve was generated for $LALF_{31-52}$ binding to LPS (A) and CLP-19 binding to LPS (B).

0.05 was considered significant and a p value less than 0.01 was considered very significant.

3. Results

3.1. Neutralization of LPS *in vitro*

The LAL test is extremely sensitive in determining the presence of free, non-neutralized LPS and even its detection of free LPS is at the pg/ml level [8]. Thus, the results of LAL test generally represent the ability of a molecule to neutralize LPS. Accordingly, the LAL test was used to assess the activity of CLP-19 to neutralize LPS. As shown in Fig. 1, CLP-19 and $LALF_{31-52}$ exhibited neutralizing activities on LPS in a dose-dependent manner with the neutralizing rates of more than 65% and 90% at the lowest and highest concentrations, respectively. Compared with positive control peptide $LALF_{31-52}$, cyclic peptide CLP-19 displayed a slightly stronger activity in LPS-neutralizing at the same concentrations.

3.2. High affinities for LPS

To examine whether the synthetic peptide CLP-19 bound specifically to LPS, FASTplot was used to generate binding curves, and K_d value was measured by FASTfit. The affinity of CLP-19 for LPS was compared with that of $LALF_{31-52}$. Data demonstrated that both peptides bound with LPS in a dose-dependent manner, which were consistent with the results of the LAL testing. CLP-19 had a lower K_d value of 8.26 μM than $LALF_{31-52}$ (47.80 μM), indicating that CLP-19 possessed the higher affinity for LPS (Fig. 2).

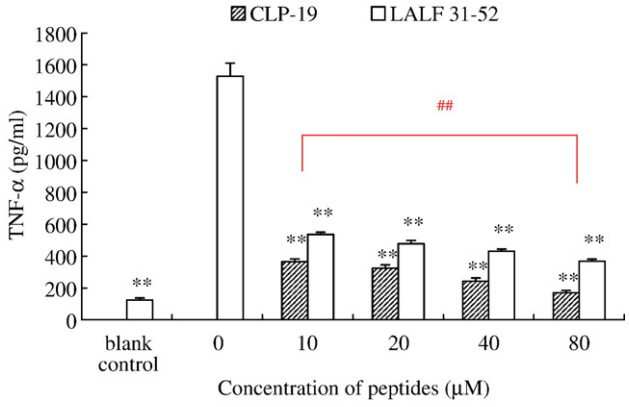

Figure 3 Peptide CLP-19 inhibited TNF-α release from LPS-stimulated RAW 264.7 cells. Mouse RAW264.7 cells (1.0×10^6 cells/ml) were pre-incubated at 37 °C for 2 h. Then 20 μl LPS (100 ng/ml) was added followed by 20 μl peptides (i.e., 10, 20, 40 and 80 μM). Incubation was carried out for another 4 h, and the levels of TNF-α in the supernatants were analyzed using the ELISA kit. Cells incubated with LPS alone and normal saline were used as positive and blank control, respectively. Values are expressed as the means±s.d. from triplicate determinations. ** $p<0.01$ compared with the LPS alone group. ## $p<0.01$, CLP-19 group compared with $LALF_{31-52}$ group.

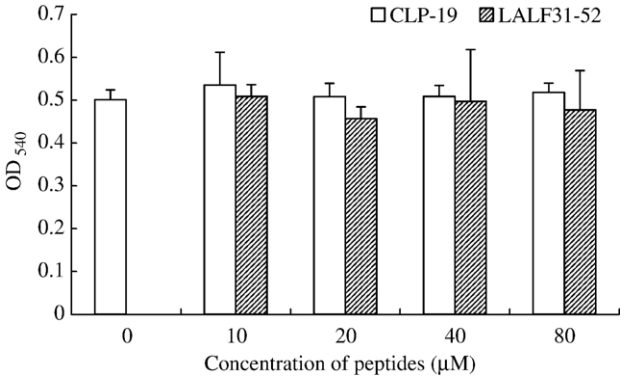

Figure 4 Cytotoxicity of peptide CLP-19 on RAW264.7 cells. Mouse RAW264.7 cells were suspended in DMEM supplemented without serum and subsequently plated in a 96-well culture dish (10^4/well). Cells were cultured at 37 °C for 2 h, and then incubated with various concentrations of the peptides for 4 h. Subsequently, 20 μl of MTT solution (5 mg/ml in PBS) were added in a total volume of 200 μl medium. After further incubation for another 4 h, the supernatant was removed and 150 μl DMSO was added to each well. The extinction was measured at 540 nm using a microplate reader.

3.3. Inhibition of TNF-α production

TNF-α is an early cytokine and a good indicator of septic shock [12–14]. To explore the potential of the two endotoxin-neutralizing peptides to inhibit TNF-α release by LPS-stimulated macrophages, we measured the TNF-α level in supernatants from mouse RAW264.7 cells incubated with LPS and peptides. Consistent with the results in LAL tests, treatment of cells with CLP-19 and $LALF_{31-52}$ both inhibited the TNF-α release in response to the presence of LPS in a dose-dependent manner (Fig. 3). Moreover, $LALF_{31-52}$ still had a lesser impact on TNF-α production than CLP-19, and this result correlated well with the ability to neutralize LPS *in vitro*.

3.4. Cytotoxicity effect of the peptides on mouse RAW264.7 cells

This *in vitro* study was investigated with MTT assay. As shown in Fig. 4, CLP-19 and $LALF_{31-52}$ were not observed to be toxic to mouse macrophages at the concentrations tested in the LPS detoxification experiments.

3.5. Protecting mice from LPS challenge

In order to investigate whether CLP-19 could protect animals from the lethal challenge of LPS, the endotoxemia mouse model was generated using a lethal dose of LPS. As presented in Fig. 5, LPS-attacked mice without either peptide treatment all died in 24 h. In contrast, it was revealed that the survival rates increased significantly ($p<0.05$) in mice injected with the peptides. More than 60% of the mice challenged by LPS and injected with various doses of CLP-19 were alive after 7 days. Similar to CLP-19, $LALF_{31-52}$ also showed excellent protective activity. Half of the animals survived on day 7, even though they were administrated $LALF_{31-52}$ with the lowest dosage of 0.025 μmol/kg.

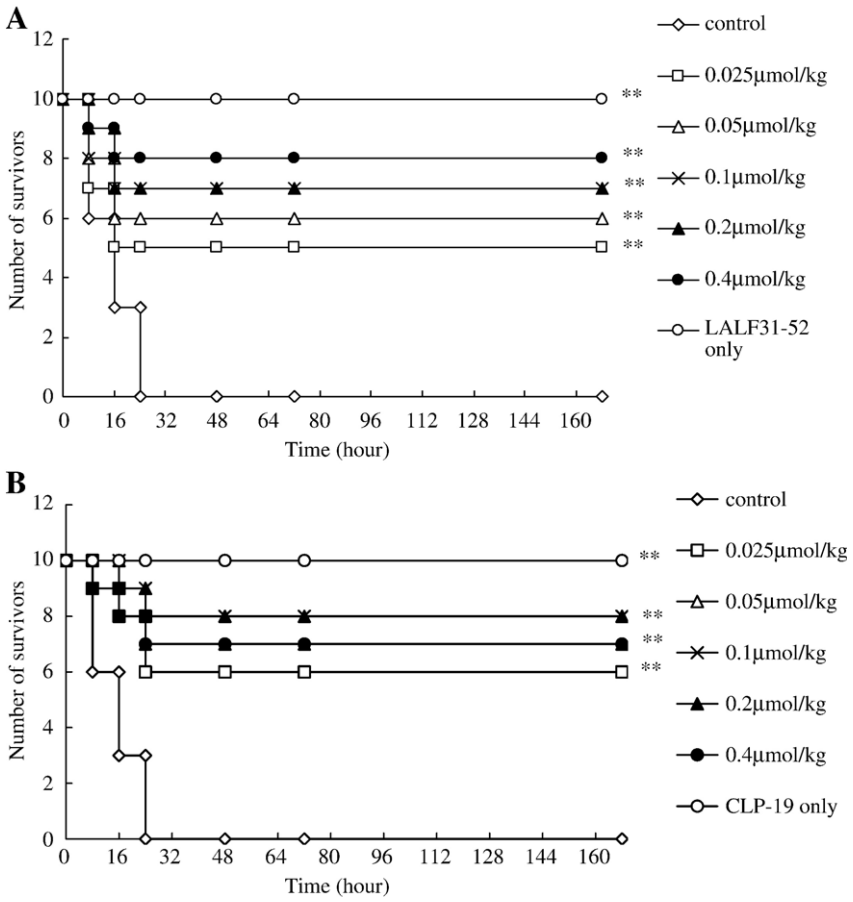

Figure 5 Survival of mice treated with peptides CLP-19 and $LALF_{31-52}$ in the endotoxemia model. Groups of mice ($n=10$) received the intra-peritoneal injection with various doses of the peptides (0.025, 0.05, 0.1, 0.2 and 0.4 μmol/kg of body weighs) in 0.1 ml saline or saline alone (0.1 ml), together with the LPS injection. (A) $LALF_{31-52}$ (B) CLP-19. LPS (20 mg/kg of body weigh in 0.1 ml saline), which produced 100% mortality in 24 h, was used to induce experimental endotoxemia. Survival was recorded every 8 h during a period of 168 h. ** $p<0.01$ compared with the control.

3.6. Suppression of TNF-α release in endotoxemia mice

To determinate whether the protective activity of CLP-19 was tightly associated with the cytokine reduction *in vivo*, we measured the serum TNF-α level in endotoxemia mice. As shown in Fig. 6, the serum TNF-α concentrations of mice injected with only peptides were almost equal to that of blank control ($p>0.05$), suggesting that the two peptides had no induction effects on TNF-α secretion in mice. There was a high level of TNF-α in serum of mice treated with only LPS. However the administration of either of the two peptides significantly decreased the production of TNF-α. The serum TNF-α levels of mice treated with the two peptides were almost equal to the blank control, and the inhibition rates of the two peptides were 91.4% and 87.4%, respectively (Fig. 6). As expected, the inhibitory activity on TNF-α release of CLP-19 was still a little superior to that of $LALF_{31-52}$.

3.7. Reducing serum endotoxin levels in mice

The serum endotoxin levels in mice were measured in order to determine whether the *in vivo* suppression of TNF-α release was closely associated with the LPS decrease. As shown in Fig. 7, both peptides showed conspicuous endotoxin-neutralizing

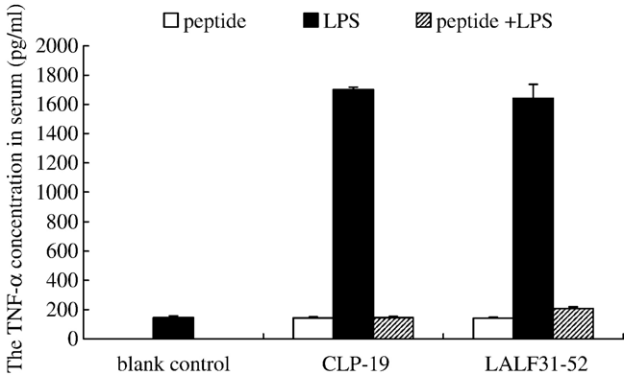

Figure 6 The inhibition of the TNF-α release by peptides CLP-19 and $LALF_{31-52}$ in mice. For each peptide, mice were intraperitoneally injected with peptide (5 mg/kg) or solutions of LPS (2 mg/kg) or mixture solution (1:1) of LPS and peptide ($n=4$). Mice given only saline were as blank control. The total injection volume was 0.2 ml per 20 g bodyweight. Sixty minutes after intra-peritoneal injection, blood samples (10 μl each) were collected from the sacrificed mice. After dilution, the level of TNF-α in the diluents was analyzed using the ELISA kits. Values are expressed as the means±s.d. from triplicate determinations. ** $p<0.01$ compared with LPS alone group.

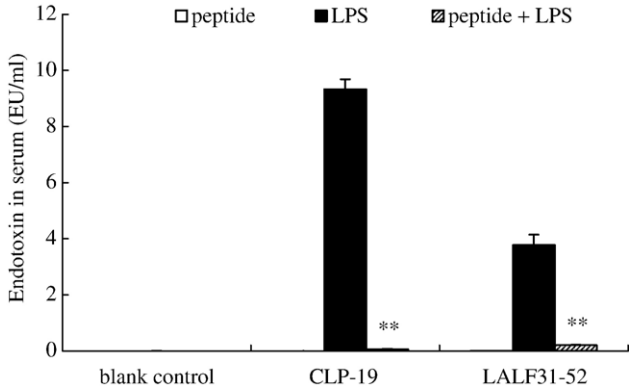

Figure 7 Peptides CLP-19 and $LALF_{31-52}$ reduced the plasma endotoxin levels in mice challenged with a lethal LPS dose. For each peptide, mice were intraperitoneally injected with peptide (5 mg/kg) or solutions of LPS (2 mg/kg) or mixture solution (1:1) of LPS and peptide ($n=4$). Mice given only saline were as blank control. The total injection volume was 0.2 ml per 20 g bodyweight. Sixty minutes after intra-peritoneal injection, blood samples (10 μl each) were collected from the sacrificed mice. The endotoxin levels in the diluents were assayed using the LAL test. Values are expressed as the means ± s.d. from triplicate determinations. ** $p<0.01$ compared with the LPS alone group.

activities in the serum of endotoximia mice. The serum endotoxin levels in mice were significantly lowered 1 h after the animals were treated with LPS-peptide mixtures, compared with the positive control. The result was consistent with that *in vitro* neutralization study, which suggested that CLP-19 was able to neutralize endotoxin not only *in vitro* but also *in vivo*.

4. Discussion

Gram-negative bacteria are the most frequent cause of septic shock. Endotoxin, a major component of the outer membrane of gram-negative bacteria, is the key to initiate the complex reaction patterns of the host [15]. Current treatments for gram-negative septic shock rely on antibiotics to control the infections and intensive-care support to correct the dysfunction of cardiovascular, respiratory and other organ systems. Nevertheless, the curative effect is yet not satisfactory and optimistic. As for LPS detoxification, the remarkable LPS antagonism of certain endotoxin-neutralizing peptides [3–7,16–18] is likely promising. Accordingly, we designed and synthesized a peptide, CLP-19, based on the structure of LALF, expecting that this synthetic peptide will be a potential agent applied in the therapy of septic shock. CLP-19 is composed of 19 amino acid residues and is head-to-tail looped via disulfide bonds of cysteine residues on two terminals. The activity of CLP-19 to antagonize *LPS in vitro* and *in vivo* was thoroughly investigated in this study. The results reveal that CLP-19 was significantly efficient in LPS-neutralizing both *in vitro* and *in vivo* (Table 1).

LPS consists of three chemical components parts: the O-specific antigen, the core oligosaccharide and lipid A, an evolutionary conserved region, which is known as the essential toxic component of LPS. The two phosphate groups of lipid A and the oligosaccharide core portion of LPS are exhibiting negative charges and, which can interact with the positive charges in cationic peptides, e.g., especially for CLP-19 (with 9 positive charges). Moreover, about 42% of amino acid residues of CLP-19 are hydrophobic that likely leads to the binding of the fatty acyl groups of lipid A to the peptide via hydrophobic interaction. It is likely that both the electrostatic attraction and the hydrophobic interaction contribute to the binding of LPS to CLP-19. Furthermore, it is known that cyclic conformation of peptides is not only important for high affinity LPS binding, but also has an advantage of conferring greater stability in plasma [9]. Therefore, it is reasonable that cyclic peptide CLP-19 exerted the markedly effective LPS-neutralizing activity as demonstrated in this study. Previous reports have shown that the binding of cationic peptides to LPS may influence the interaction between LPS and its receptors, e.g., LBP and CD14, resulting in blocking the LPS-triggering intracellular signaling [19–21]. Therefore, the binding to LPS is likely the base of the mechanism of endotoxin detoxification for CLP-19.

In this study, we used the LAL test to assess the LPS-neutralizing activity of CLP-19 both *in vitro* and *in vivo*. Although the assay is sensitive enough to evaluate the activity of molecules to neutralize LPS *in vitro*, many factors may influence the LAL test, such as acetic acid and heparin [22,23]. The biosensor method that was used does not suffer from the interference of these compounds, and therefore exhibits an advantage over the LAL assay. As shown in Fig. 2, the K_d value of CLP-19 was lower than $LALF_{31-52}$, suggesting a higher LPS-binding activity of CLP-19 than that of the control peptide. The abilities of the two peptides to neutralize LPS *in vitro*, measured by LAL test, were almost at the same level. However, the K_d value of CLP-19 is nearly one sixth of that of $LALF_{31-52}$. Obviously there is a large disparity between the two K_d values, which usually indicates that there has certain difference in the binding abilities to LPS between the two peptides. This result was not in accord with that in LAL assay, suggesting that for the evaluation of endotoxin-neutralizing activities of peptides, it is better to use the LAL assay together with the biosensor method.

The development of efficient anti-LPS peptides requires that not only they have specific binding or neutralizing

Table 1 The characterization of the peptides used in this study

Peptide	Amino acid composition	Molecular Weight (Da)	Purity (%)	Description
CLP-19	CRKPTFRRLKWKIKFKFKC	2511.1	94.4	Cyclic peptide
$LALF_{31-52}$ [a]	CHYRIKPTFRRLKWKYKGKFWC	2945.5	85.9	Linear peptide

[a] The peptide LALF31-52 was synthesized with the Symphony Peptide Synthesis (Protein Technologies, Inc., USA) utilizing Fmoc chemistry via stepwise solid phase peptide assembly starting from a Fmoc-Lys (Boc)-Wang resin and purified by HPLC (prepared by Shenzhen Hybio Engineering, Shenzhen, China).

activities *in vitro*, but that they also prove efficacious in endotoxemia animal models. Accordingly, the anti-endotoxin effects of CLP-19 on mice challenged with a lethal dose of LPS were further examined. After CLP-19 treatment, the survival rates of mice received lethal LPS challenge were high (60–80% within 7 days), similar to those of $LALF_{31-52}$ group (50–80%). In addition, the serum levels of TNF-α significantly decreased after CLP-19 administration, associated with the serum LPS reduction. These parallel results probably revealed that the endotoxin-neutralizing activity of CLP-19 is responsible for the decrease of TNF-α production *in vivo*, resulting in obvious protective effect on LPS-attacked mice, which was characterized by the increasing survival. This may be an important mechanism of CLP-19 that protects animals challenged by LPS. Moreover we found that CLP-19 could block the binding of LPS to LBP and consequently inhibit the TNF-α release in response to LPS *in vitro* (unpublished data), the results indicated that the ability of LPS–LBP binding blockage of CLP-19 may be one of the possible mechanisms of LPS antagonism, but whether this mechanism works and what role of it plays *in vivo* will be verified in further study. Together, this study clearly suggests that CLP-19 is a potential candidate as a clinical anti-endotoxin agent and warrants further study.

Acknowledgments

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