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New Antiseptic Peptides To Protect against Endotoxin-Mediated Shock▽

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Systemic bacterial infections are associated with high mortality. The access of bacteria or constituents thereof to systemic circulation induces the massive release of immunomodulatory mediators, ultimately causing tissue hypoperfusion and multiple-organ failure despite adequate antibiotic treatment. Lipid A, the "endotoxic principle" of bacterial lipopolysaccharide (LPS), is one of the major bacterial immunostimuli. Here we demonstrate the biological efficacy of rationally designed new synthetic antilipopolysaccharide peptides (SALPs) based on the *Limulus* anti-LPS factor for systemic application. We show efficient inhibition of LPS-induced cytokine release and protection from lethal septic shock *in vivo*, whereas cytotoxicity was not observed under physiologically relevant conditions and concentrations. The molecular mechanism of LPS neutralization was elucidated by biophysical techniques. The lipid A part of LPS is converted from its "endotoxic conformation," the cubic aggregate structure, into an inactive multilamellar structure, and the binding affinity of the peptide to LPS exceeds those of known LPS-binding proteins, such as LPS-binding protein (LBP). Our results thus delineate a novel therapeutic strategy for the clinical management of patients with septic shock.

The life-threatening clinical consequences of sepsis and septic shock arise from recognition of microbial immunostimulatory molecules by the hosts' professional immune cells and the release of hemodynamically active mediators. The most potent immunostimulatory constituents are part of the microbial cell envelope, such as lipopolysaccharide (LPS) or lipoproteins. They are released continuously due to cell growth and division and massively liberated as a consequence of the attack of the immune system. In the case of Gram-negative bacteria, the most potent factor is LPS, which, therefore, is also called an endotoxin. LPS concentrations in blood serum as low as 1 ng/ml are able to cause sepsis. Septic shock resulting from bacterial infection remains a frequent cause of death, particularly in intensive care units, with more than 200,000 people dying each year in the United States alone. Death by septic shock can happen despite appropriate broad-range antibiotic treatment, which may kill bacteria but is not only incapable of neutralizing immunostimulatory LPS but also may promote its release into circulation (11).

The response of mammalian cells to LPS is initiated by its interaction with serum proteins such as lipopolysaccharide-binding protein (LBP) and specific receptors and/or binding proteins of immune cells such as soluble CD14 (sCD14) and membrane-bound CD14 (mCD14), which finally leads to cell activation through the Toll-like receptor 4 (TLR4)–MD-2 pathway (31). The hydrophobic moiety of LPS, lipid A, anchoring LPS to the bacterial outer membrane, constitutes the "endotoxic principle" of LPS (24). Enterobacterial lipid A consists of a diglucosamine backbone phosphorylated at positions 1 and 4′, to which six acyl chains are linked at positions 2,3 and 2′,3′. The physicochemical properties of LPS suggest that cationic amphiphilic agents could effectively neutralize its lipid A moiety. A number of studies have used natural proteins, peptides, or modified variants thereof to neutralize LPS and, thus, to protect it from endotoxin-mediated immunostimulation (1, 10), and in some cases, a significant protection from LPS-induced lethality *in vivo* was observed (10, 19). However, these approaches required very high peptide/LPS molar ratios, precluding their use in humans due to the intrinsic cytotoxicity of the peptides at these concentrations.

We have designed a completely new class of peptides—synthetic anti-LPS peptides (SALPs). SALPs were originally based on the LPS-binding domain of the *Limulus* anti-LPS factor (LALF) (2) but were substantially changed in length and primary sequence for optimal binding to the lipid A portion of LPS. Here, we report that these peptides are highly efficient in neutralization of LPS and blockage of its immunopathological consequences *in vitro* and *in vivo*. Our preclinical study reveals that these SALPs combine excellent selectivity for LPS, with high neutralizing activity *in vitro* and potent protection to septic shock using the murine model *in vivo*. We also demonstrate very low cytotoxicity under physiological conditions, making these SALPs promising candidates for their application as therapeutic agents for the prevention and treatment of septic shock. The molecular interaction mechanism between

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SALPs and LPS was studied using various biophysical approaches.

MATERIALS AND METHODS

Lipids. Lipopolysaccharides from the rough mutants Re and Ra from *Salmonella enterica* serovar Minnesota (R595 and R60, respectively) were extracted by the phenol-chloroform/petrol ether method (13) from bacteria grown at 37°C, purified, and lyophilized. Results from all the standard assays performed on the purified LPS (analysis of the amount of glucosamine, total and organic phosphate, and the distribution of the fatty acid residues) were in good agreement with the chemical properties expected for the LPS chemotypes, whose molecular structures have already been solved (23). S-lipopolysaccharides of *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* PAO1 for mouse testing were obtained from the aqueous phase of a water-phenol extract and purified by treatment with chaotropic agents and detergents, according to published procedures (14, 17).

Preparation of endotoxin aggregates. LPS was solubilized in the appropriate buffer (lipid concentration of 1 to 10 mM, depending on the applied technique), extensively vortexed, sonicated for 30 min in a water bath, and subjected to several temperature cycles between 20 and 60°C. Finally, the lipid suspension was incubated at 4°C for at least 12 h before use.

Peptide synthesis. The peptides were synthesized with an amidated C terminus by the solid-phase peptide synthesis technique in an automatic peptide synthesizer (model 433A; Applied Biosystems) on Fmoc-Rink amide resin, according to the 0.1-mmol FastMoc synthesis protocol of the manufacturer, including the removal of the N-terminal Fmoc group. The peptidyl resin was deprotected and cleaved with a mixture of 90% trifluoroacetic acid (TFA), 5% anisole, 2% thioanisole, and 3% dithiothreitol for 90 min at room temperature. After cleavage, the suspension was filtered through a syringe filter into ice-cold diethyl ether. The precipitated peptides were separated by centrifugation and repeatedly washed with cold ether. The final purification was done by reverse-phase high-pressure liquid chromatography (RP-HPLC). Purity levels of up to 98% were achieved by using an Aqua C_{18} column (Phenomenex) in combination with dedicated gradients of acetonitrile in 0.1% TFA, checked by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy and RP-HPLC at 214 nm. The sequences of the peptides were submitted in an international patent, which was published in October 2009 by the European Patent Office (patent 2108 372 A1).

Isothermal titration calorimetry (ITC). Microcalorimetric experiments of peptide binding to LPS were performed on a MSC isothermal titration calorimeter (MicroCal Inc., Northampton, MA) at 40°C. Briefly, after thorough degassing of the samples, peptide (1 to 4 mM in 20 mM HEPES, pH 7.0) was titrated to an LPS suspension (0.05 mM in 20 mM HEPES, pH 7.0). The enthalpy change during each injection was measured by the instrument, and the area underneath each injection peak was integrated (Origin; MicroCal) and plotted against the molar ratio of the concentrations of peptide to LPS. Titration of the pure peptide into HEPES buffer resulted in a negligible endothermic reaction due to dilution, which was subtracted from the plotted curves. The experiments were done at least twice.

Small-angle X-ray scattering (SAXS) with synchrotron radiation. X-ray scattering measurements of mixtures of lipid A with Pep19-2.5 were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation facility HASYLAB using the double-focusing monochromator-mirror camera X33 (16). Diffraction patterns in the range of the scattering vector $0.001 < s < 0.08$ Å^{-1} (where s is $2 \sin \theta/\lambda$, 2θ is the scattering angle, and λ is the wavelength, which was 1.5 Å) were recorded at 20, 40, and 60°C, with exposure times of 1 min using an image plate detector with online readout (mar345; Marresearch, Norderstedt, Germany) (26). The s axis was calibrated with Ag-behenate, which has a periodicity of 58.4 Å. The diffraction patterns were evaluated as described previously (6), assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures. The lamellar and cubic structures are most relevant here and are characterized by the following features. (i) Lamellar structures: the reflections are grouped in equidistant ratios, i.e., 1, 1/2, 1/3, 1/4, etc. of the lamellar repeat distance d_l. (ii) Cubic structures: the different space groups of these nonlamellar three-dimensional structures differ in the ratio of their spacings. The relationship between reciprocal spacing $s_{hkl} = 1/d_{hkl}$ and lattice constant a is $s_{hkl} = [(h^2 + k^2 + l^2)/a]^{1/2}$ (hkl = Miller indices of the corresponding set of plane).

Stimulation of human MNC by LPS. Mononuclear cells (MNC) were isolated from heparinized blood samples obtained from healthy donors as described previously (15). The cells were resuspended in medium (RPMI 1640), and their number was equilibrated at 5×10^6 cells/ml. For stimulation, 200 μl MNC (1×10^6 cells) was transferred into each well of a 96-well culture plate. LPS Ra (from *S.* Minnesota strain R60) and the LPS/peptide ratio mixtures were preincubated for 30 min at 37°C and added to the cultures at 20 μl per well. The cultures were incubated for 4 h at 37°C with 5% CO_2. Supernatants were collected after centrifugation of the culture plates for 10 min at $400 \times g$ and stored at −20°C until immunological determination of tumor necrosis factor alpha (TNF-α), carried out with a sandwich enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody against TNF (clone 6b; Intex AG, Switzerland) and described previously in detail (15).

Cytotoxicity assays and hemolysis. Cytotoxicity was assayed by using the chip-based Bionas system with four different cell types. These include human hepatoma cells (HepG2), human colon adenocarcinoma cells (LS-174T), human acute lymphocytic leukemic cells (Jurkat), and human peripheral blood mononuclear cells (hPBMC), provided by Cell Line Services (Piscataway, NJ) and ProBioGen (Berlin, Germany). The cells were seeded on chips, with a density of 200,000 cells/chip for HepG2 and LS-174T, 300,000 cells/chip for Jurkat, and 2 million cells/chip for PBMC.

Red blood cells (RBCs) were obtained from citrated human blood by centrifugation ($1{,}500 \times g$, 10 min), washed three times with isotonic 20 mM phosphate-NaCl buffer (pH 7.4), and suspended in the same buffer at a concentration equivalent to 5% of the normal hematocrit. Forty-microliter aliquots of this RBC suspension were added to 0.96 ml of peptide dilutions prepared in the same isotonic phosphate solution, incubated at 37°C for 30 min, and centrifuged ($1{,}500 \times g$, 10 min). The supernatants were analyzed spectrophotometrically (with absorbance at 543 nm) for hemoglobin, and results were expressed as the percentage released with respect to sonicated controls (100% release) or controls processed without peptides (0% release) (21).

Assays for antibacterial activities. The antibacterial activity of the peptides was determined by microdilution susceptibility assays performed in standard Mueller-Hinton (MH) broth. Susceptibility testing was performed following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (20). Briefly, serial 2-fold dilutions of the peptides were made in MH broth and dispensed into 96-well U-bottom polystyrene microtiter plates. Bacterial cells grown for 18 to 20 h on agar plates were suspended in saline, and the turbidity was adjusted to an optical density at 600 nm (OD_{600}) of 0.04 (1×10^7 CFU/ml, approximately). A 100-fold dilution of this suspension prepared in MH broth was mixed 1:1 with 100 μl of each of the corresponding peptide dilutions. Plates were incubated at 37°C without shaking for 18 to 20 h, and the MIC was determined visually.

Animal model of endotoxicity. As a compound for eliciting endotoxic shock, LPS from *Pseudomonas aeruginosa* PA01 or from *S.* Minnesota strain R60 was used.

Female C57/BL6 mice weighing 14 to 16 g (6 weeks old) were purchased from Harlan Spain (Harlan Interfauna Iberica S.A., Barcelona, Spain) and randomly distributed in experimental groups ($n = 8$). Endotoxic shock was induced in the animals by coinoculation of LPS and galactosamine by following the method of Galanos et al. (12). Specifically, each animal received an intraperitoneal injection containing a mixture of 25 ng of LPS from *S.* Minnesota R60 (or 150 ng when using *P. aeruginosa* LPS) and 18 mg of galactosamine resuspended in 200 μl of endotoxin-free saline.

Immediately after LPS administration, animals were intraperitoneally inoculated with 12.5 or 1.25 μg (150 μg in the case of animals receiving *P. aeruginosa* LPS) of the test peptide resuspended in 150 μl of pyrogen-free saline. To facilitate the therapeutic action of the peptide, mice so treated were gently massaged at the site of inoculation for a few seconds. Animal mortality was monitored at 6 h and 24 h postinoculation and at daily intervals for 7 days.

In each independent experiment, a group of animals received 150 μl of pyrogen-free saline containing 12.5 μg of polymyxin B (PMB), a lipopeptide with well-known antiendotoxic properties, whereas another group was left untreated.

The results of animal mortality at all experimental time points were globally analyzed using Kaplan-Meier survival analysis (SPSS 15.0). When the survival plots were parallel, data were compared by the log rank test, whereas for those plots that intersected, the Breslow-Gehan-Wilcoxon test was applied. P values were always obtained by comparing data from the same experiment (mortality in treated versus untreated groups).

All the animal protocols used in this study were approved by the University of Navarra Animal Research Committee (protocol 035/05).

RESULTS

Design of the SALP. The sequences of the Pep19 series based on the *Limulus* anti-LPS factor, presented in Fig. 1A,

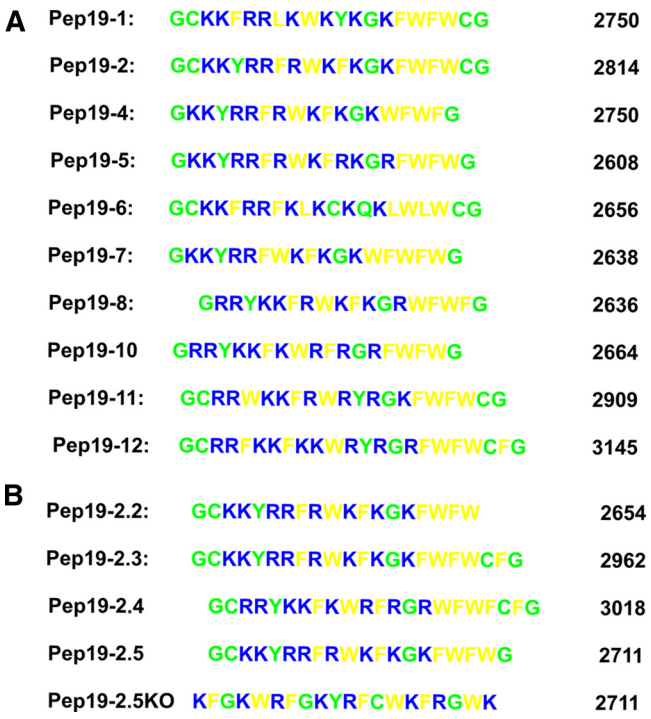

FIG. 1. Amino acid sequence of Pep19-derived peptides. (A) Sequences of the basic peptides Pep19-1 to Pep19-12 and their molecular weights (MW). (B) Sequences of the Pep19-2 series. Amino acids: G, glycine; C, cysteine; Q, glutamine; Y, tyrosine; R, arginine; K, lysine; F, phenylalanine; W, tryptophan; L, leucine. The colors illustrate the main physicochemical characteristics, as follows: green, polar; yellow, hydrophobic; blue, basic (positively charged).

were designed according to the following physicochemical criteria. (i) The compounds require amphiphilic characters, i.e., they must contain sufficiently high numbers of polar and positively charged (basic) amino acids and corresponding hydrophobic residues. (ii) The number of basic (positively charged) amino acids must be sufficiently high to bind to the negatively charged moieties of the LPS backbone. For this, 7 or 8 arginine (R) or lysine (K) residues were selected. The hydrophilic cationic part of the peptides was localized mainly near the N-terminal residue, and the hydrophobic moiety was localized mainly near the C-terminal end. The number of the hydrophobic residues, consisting essentially of tryptophan (W) and phenylalanine (F), was 6 or 7. (iii) The length of the peptide should be optimized to account for the length and character of the lipid A moiety in such a way that the hydrophobic moiety of the peptide readily intercalates into the hydrocarbon chains of lipid A and the cationic moiety intercalates into the backbone-adjacent oligosaccharide moiety of LPS. A medium length of 19 amino acids was assumed to represent an optimum, but a maximum number of 23 amino acids was allowed. (iv) On the basis of the best sequences, as judged by their ability to inhibit cytokine production in human mononuclear cells (see below), new peptide variants were synthesized. To this end, the sequence of compound Pep19-2 was used as a template, giving rise to further peptides Pep19-2.2 to Pep19-2.5 (Fig. 1B).

Antibacterial activity. The antibacterial activity of the peptides was assessed on strains of various clinically relevant bacteria (Table 1). The data clearly show extreme differences in their MICs depending sensitively on the amino acid sequences. The SALPs showed activity not only against Gram-negative bacteria but also against Gram-positive bacteria. The comparison shows that the GC motif at the N-terminal end and the CG motif at the C-terminal end seem to be rather disadvantageous, since compounds lacking the glycine moieties were more active. Furthermore, peptides partially lacking that motif, such as Pep19-8, had increased potency against 2 methicillin-resistant *Staphylococcus aureus* (MRSA) strains, with MIC values around 8 μg/ml.

Inhibition of cytokine production in human mononuclear cells. The LPS-induced production of tumor necrosis factor alpha (TNF-α) by human mononuclear cells at three LPS concentrations (100, 10, and 1 ng/ml) and its inhibition by some selected peptides (Pep19-2 and Pep19-8) at different LPS/peptide weight ratios are plotted in Fig. 2A. Both peptides inhibit the LPS-induced TNF-α secretion to different degrees, showing for Pep19-2 the highest efficacy by far at a Pep19-2/LPS concentration ratio ([Pep19-2]/[LPS] ratio) of 100:1 weight percent (corresponding to a molar ratio of 65:1). Similar results were found for Pep19-1 (data not shown). In contrast to these compounds, Pep19-8 was much less active in the inhibi-

TABLE 1. MICs of peptides on indicated bacterial strains

Peptide	MIC (μg/ml)[a]								
	Bordetella bronchiseptica CUN 11844-99	*P. aeruginosa* CUN 4158-02	*P. aeruginosa* ATCC 27853	*E. coli* ATCC 25922	*S. aureus* ATCC 25923	MRSA ATCC 43300	MRSA CUN 3792-99	*Stenotrophomonas maltophilia* CUN 3998-00	*Acinetobacter baumannii* ATCC 19606
Pep19-1	4	256	256	64	32	64	1,024	512	64
Pep19-2	32	256	128	128	128	128	1,024	512	64
Pep19-4	4	16	16	16	16	16	16	32	16
Pep19-5	4	32	16	16	16	16	16	64	16
Pep19-6	16	256	256	1,024	1,024	64	32	1,024	64
Pep19-7	8	32	8	1,024	1,024	16	16	64	16
Pep19-8	128	32	16	16	8	8	8	32	16
Pep19-10	16	ND	ND	64	32	ND	ND	ND	ND
Pep19-11	32	128	128	128	64	32	64	64	64
Pep19-12	32	128	128	64	64	32	64	64	64
Pep19-2.2	64	128	64	64	64	32	64	128	64

[a] CUN, Clinica Universidad de Navarra (University Hospital of Navarra); MRSA, methicillin-resistant *Staphylococcus aureus*; ND, not done.

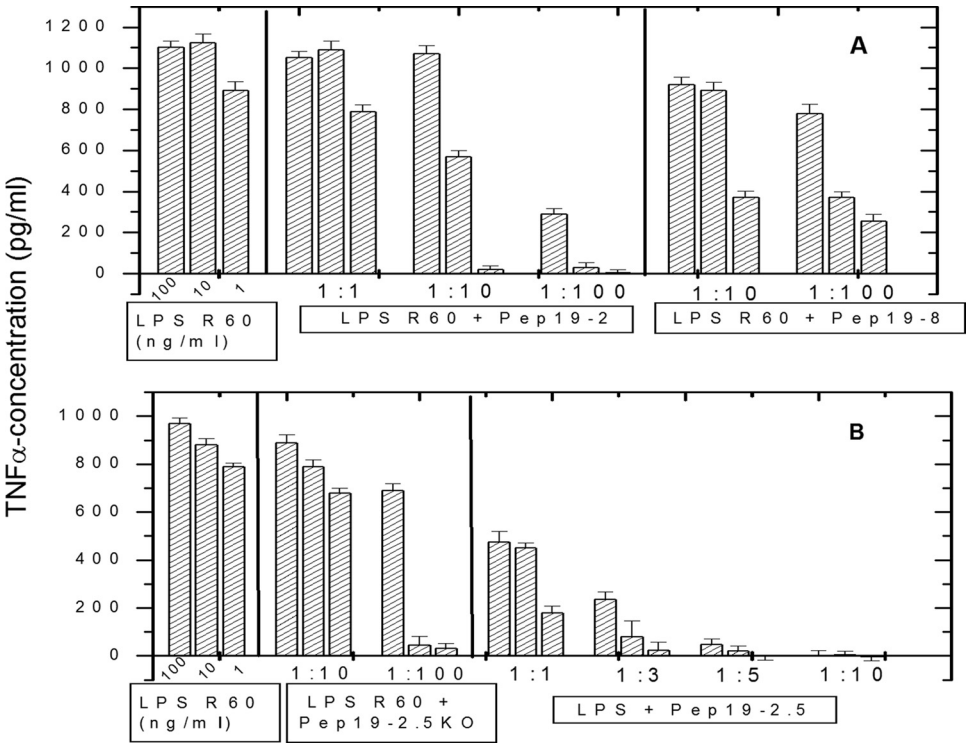

FIG. 2. Inhibitory effect on LPS-induced cytokine release. LPS R60-induced secretion of TNF-α by human mononuclear cells and the inhibitory effect in the presence of the peptides Pep19-2 and Pep19-8 (A) and Pep19-2.5 and Pep19-2.5KO (Pep19-2.5 control) (B). The peptides were added at the indicated weight ratios of the concentrations of LPS to peptide.

tion of the LPS response (Fig. 2A), and a similar observation was made for Pep19-5 (data not shown).

On the basis of these and other results on the inhibition of the cytokine production, peptides containing variations of the amino acid sequences were designed using Pep19-2, in particular, as a template, as shown in Fig. 1B. Data from the inhibition assay are presented in Fig. 2B, showing the extreme activity of Pep19-2.5 to inhibit the production of TNF-α induced by LPS at all investigated concentrations. Already at a [Pep19-2.5]/[LPS] ratio of 3:1 weight (corresponding to a molar ratio of 1.9:1), there is nearly complete inhibition. This was similarly true for Pep19-2.2 (data not shown). Comparative measurements with polymyxin B (PMB), the gold standard of antimicrobial peptides, showed that PMB causes some inhibition of the cytokine secretion at a [PMB]/[LPS] ratio of 10:1 weight (corresponding to a molar ratio of 3:1) but no inhibition at lower peptide concentrations. This means that the best peptides of the Pep19 series significantly outperform the LPS-neutralizing activity of the gold standard.

A variant of Pep19-2.5, with its same amino acid composition but in a completely random sequence, called Pep19-2.5KO was shown to still exert some inhibition but considerably lower than that of the parent peptide (Fig. 2A).

Animal model of endotoxicity. To investigate whether the *in vitro* data could be extended to an *in vivo* model of endotoxicity, we evaluated the ability of various peptides to protect mice against a lethal septic shock induced by the intraperitoneal injection of LPS. Initially, *Pseudomonas aeruginosa* LPS was used, harboring an endotoxic activity that is known to be lower than that of *Salmonella* (see Materials and Methods). As shown in Fig. 3A, the *in vivo* results, performed at a [peptide]/[LPS] ratio of 1,000:1 weight (corresponding to a molar ratio of approximately 600:1) were in good agreement with the *in vitro* data, since the peptides with less inhibitory activity (Pep19-5 and Pep19-8) did not confer protection against LPS-induced shock, whereas highly active compounds such as Pep19-2, Pep19-12, and Pep19-2.2 protected the animals as efficiently as polymyxin B.

In addition, the ability of the peptides to neutralize LPS of enhanced endotoxicity was examined. Pep19-2.2 and Pep19-2.5 were selected, and the experimental setting was repeated using LPS from *S. enterica* serovar Minnesota R60. As shown in Fig. 3B, administration of Pep19-2.5 conferred a significant level of protection, similar to that of polymyxin B, whereas Pep19-2.2 was slightly less protective. Interestingly, animals receiving Pep19-2.5 at doses 1 order of magnitude lower (2.5 μg, i.e., a ratio of 1:50 weight percent, with respect to LPS) than the previous one were fully protected during the first 48 h after inoculation, although the therapeutic effect decreased slightly at later time points

Cytotoxicity and hemolysis. To test possible side effects of the peptides in physiologically relevant cells, several parameters, including respiration rate, acidification, and impedance, were monitored kinetically in a chip-based system (Bionas) at concentrations of 0.01, 0.1, 1, 10, and 100 μg/ml. In Fig. 4A, data for the respiration rates are shown for Jurkat cells, which clearly give evidence that no damaging effect can be observed at concentrations of up to 10 μg/ml. At 100 μg/ml Pep19-2.5,

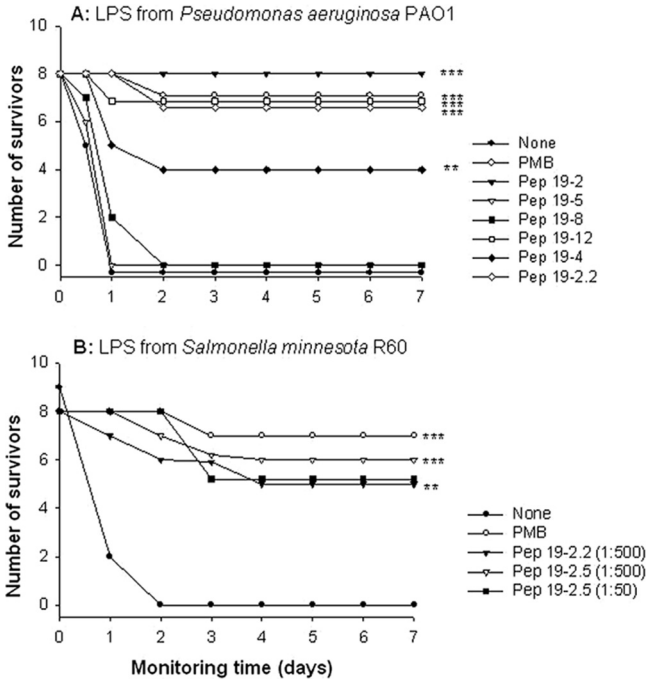

FIG. 3. Protective effect in an *in vivo* endotoxin exposure model. (A) Protection against lethal septic shock caused by injection of *Pseudomonas aeruginosa* LPS. On day 0, a group of C57BL/6 mice ($n = 8$) was inoculated intraperitoneally with a mixture of 18 mg galactosamine and 150 ng LPS isolated from *Pseudomonas aeruginosa* PAO1. Immediately afterward, animals received 150 μg of the test peptide or 150 μg of polymyxin B at a different site of the peritoneum. Animal mortality was monitored at daily intervals for 7 days. Statistical differences were analyzed by the Kaplan-Meier survival test (***, $P < 0.001$; **, $P < 0.01$). (B) *S.* Minnesota LPS. On day 0, a group of C57BL/6 mice ($n = 8$) was inoculated intraperitoneally with a mixture of 18 mg galactosamine and 25 ng LPS from *S.* Minnesota R60. Immediately afterward, animals received either 12.5 μg or 1.25 μg (1:500 and 1:50 weight ratios, with respect to LPS) of the test peptide or polymyxin B at a different site of the peritoneum. Animal mortality was monitored at daily intervals for 7 days. Statistical differences were analyzed by the Kaplan-Meier survival test (***, $P < 0.001$; **, $P < 0.01$).

however, the cells are irreversibly damaged. These observations were found to be true for all 4 different cellular systems, namely, human hepatoma cells (HepG2), human colon adenocarcinoma cells (LS-174T), human acute lymphocytic leukamic cells (Jurkat), and human peripheral blood mononuclear cells (hPBMC). Similar results were also found for HeLa and HaCaT cells using conventional test systems such as the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test (data not shown).

Using the red blood cell hemolysis assay for selected peptides, no essential damage was observed at peptide concentrations of up to 25 μg/ml (ca. 10 μM) (Fig. 4B).

Binding experiments. To understand the mechanisms of binding, high-sensitivity isothermal titration calorimetry (ITC) was applied by repeated titration of the peptide solution into the LPS dispersion. The experiments show a strong exothermic reaction due to Coulomb attraction between Pep19-2.5 and LPS R60 (peaks downward), which runs into saturation already at a [Pep19-2.5]/[LPS] ratio of 0.3 (Fig. 5). This means that already 3 molecules of the peptide, corresponding to 24 positive charges, are sufficient to neutralize 10 LPS molecules (40 negative charges). Due to the amphiphilic character of the peptide, saturation takes place before charge compensation.

Supramolecular aggregate structure by SAXS. It is known that the bioactive aggregate structure of LPS or lipid A is cubic (7). The influence of Pep19-2.5 on the aggregation properties of lipid A was tested, because the scattering patterns of this endotoxin species with its short sugar chain provide the best resolution. In Fig. 6, the data are shown for pure lipid A (panel A) and in the presence of a small amount of Pep19-2.5 (panel B; [lipid A]/[Pep19-2.5] molar ratio of 7:1). Lipid A alone expresses a complex spectrum with cubic symmetry, because the reflections at 8.18, 5.29, and 4.06 nm obey the relations $a_Q/\sqrt{2}$, $a_Q/\sqrt{5}$, and $a_Q/\sqrt{8}$, respectively, with a cubic periodicity of a_Q equaling 11.6 ± 0.2 nm. In the presence of the peptide, there is a complete conversion of the spectrum with the appearance of one strong peak at 5.07 nm, corresponding to the lamellar repeat characteristic of lipid A with multilamellar structure (7).

DISCUSSION

We have designed and synthesized cationic amphiphilic peptides (synthetic antilipopolysaccharide peptides [SALPs]) which are able to neutralize bacterial endotoxins effectively in an *in vitro* system—human mononuclear cells—as well as *in vivo* in a murine animal model of endotoxicity. The best peptides inhibit the production of TNF-α in human mononuclear cells already at a slightly excess weight percent or molar ratio of the concentrations of peptide to LPS (Fig. 2A and B), making them suitable candidates for antiseptic agents. This was clearly demonstrated by employing the animal model of Gram-negative sepsis, exhibiting considerable protection against endotoxic shock (Fig. 3) even at a relatively low peptide dose. Regarding the latter result, one has to take into account that in the therapeutic situation, the peptide would be administered several times and not only once.

At the same time, neither hemolytic nor cytotoxic effects in human hepatoma cells, human colon adenocarcinoma cells, human acute lymphocytic leukamic cells (Fig. 4A), and human peripheral blood mononuclear cells (hPBMC) or in human red blood cells (Fig. 4B) was observed for doses of up to 10 μg/ml. For an assessment of these data in relation to the situation found in human septic patients with endotoxemia, it has to be considered that under these conditions, blood serum levels of 1 ng/ml of LPS are observed (5). Both the *in vitro* and *in vivo* test systems indicate that an excess [peptide]/[LPS] ratio of 100:1 weight percent is enough to inactivate LPS effectively. This corresponds to a patient dose of 100 ng/ml, which is far below any cytotoxically relevant concentration. According to the cytotoxicity data, even a 10-fold-higher dose should not be a problem, making these peptides potentially suitable candidates for use with critical care unit patients. It is known that severe sepsis or septic shock may arise from infections due to Gram-negative bacteria in approximately 50% of all septic patients (30). Therapy with conventional antibiotics may even worsen this outcome, since many antimicrobial agents kill bacteria with subsequent release of LPS but do not inactivate LPS (11). Thus, a possible therapeutic strategy would be the use of

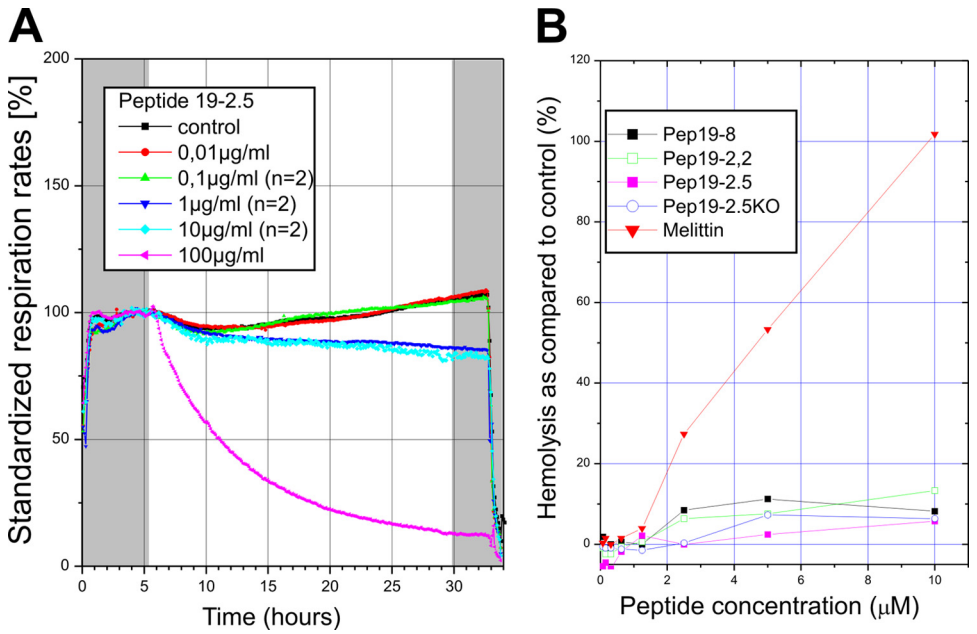

FIG. 4. Characterization of peptide-associated cytotoxicity. (A) Respiration rates of Jurkat cells over 32 h at different concentrations of peptide Pep19-2.5 in a chip-based cellular system. The first and last 5 h (gray regions) correspond to the lag and regeneration phases, respectively. (B) Red blood cell hemolysis of selected peptides compared to that of melittin from bee venom (synthetic).

the peptides presented here, since these have nonnegligible antibacterial activity levels (examples given in Table 1). In case the antimicrobial action of these compounds was not sufficient for clinical purposes, a therapeutic approach should involve the combination of peptides and antibiotics.

In Table 1 and Fig. 2A, in particular for Pep19-2 and Pep19-8, it becomes clear that the presented peptides differ in their ability to neutralize LPS in isolated forms and as constituents of Gram-negative bacteria. Pep19-2 is highly active against free LPS but has very low activity against bacteria, whereas the situation for Pep19-8 is the opposite. Therefore, the specific arrangement of the amino acids plays a decisive role, since both peptides have 8 cationic amino acids (R and L) and 7 hydrophobic amino acids (F and W) at similar chain positions. For strong anti-LPS activity, apparently the cysteine

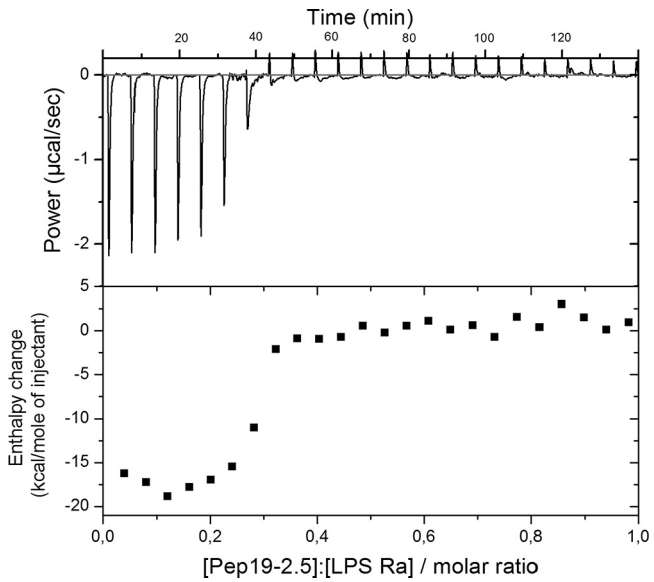

FIG. 5. Enthalpy of the peptide-LPS binding. Isothermal calorimetric titration of a 1 mM Pep19-2.5 solution into a 0.05 mM LPS Ra dispersion (from *S.* Minnesota R60). Every 5 min, 5 µl of the peptide was titrated to the LPS dispersion, and the heat reaction was recorded. A downward peak corresponds to an exothermic reaction, and an upward peak corresponds to an endothermic reaction.

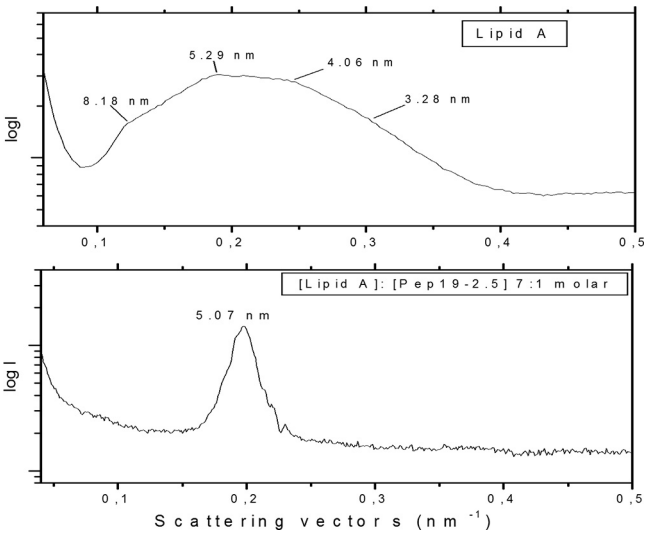

FIG. 6. Effect of Pep19-2.5 on the supramolecular lipid A aggregate structure. Synchrotron radiation small-angle X-ray scattering patterns of lipid A (top) and the [lipid A]/[Pep19-2.5] ratio of 7:1 M/M (bottom). The logarithm of the scattering intensity is plotted versus the scattering vector s ($s = 1/d$, with d being the spacings of the reflections).

in position 2 at the N-terminal end is essential, whereas this residue is irrelevant for high antimicrobial activity of all peptides (Pep19-4, -5, and -8) (Table 1). The selectivity of the peptides to inactivate LPS could be due to the distinct geometry of LPS, either in free form within a cubic aggregate structure (8) or as a constituent of the outer membrane leaflet. For these different conformations, the structural prerequisites of the peptides to neutralize LPS differ.

Differences between antimicrobial and anti-LPS activities have also been described for peptides with K_6L_9 sequences (28). Those authors emphasize that peptides with high endotoxin neutralizing activity levels are able to disaggregate the LPS multimers, deduced using fluorescence dequenching and electron microscopy (EM) with negative staining (27). In previous work on various peptides derived from the *Limulus* anti-LPS factor (2), human granulysin-derived peptides (9), human lactoferricin (4), and porcine NK2 (3), however, we found that LPS neutralization is accompanied with a high increase in endotoxin aggregation, leading to multilamellar structures (Fig. 6), as also shown by freeze-fracture EM (2). Freeze-fracture EM is more adequate than EM with negative staining, with the latter changing the morphology of LPS due to the change in water content, as demonstrated by Risco and coworkers (25). Also, differences in the LPS preparation used (Rosenfeld et al. used the LPS S form from *E. coli* O111:B4 [29]) cannot account for distinct aggregation properties, since the peptides investigated here are equally efficient at inhibiting the cytokine production induced by LPS from several rough and smooth bacterial strains (LPS Re, LPS Rc, and LPS Ra, all from *S. enterica* serovar Minnesota; LPS S form from *Salmonella abortusequi*; LPS S form from *E. coli* 08:K27; and LPS from *Chromobacterium violaceum*) (data not shown).

The decrease in LPS bioactivity with the increased size of LPS aggregates can be explained by the fact that the binding sites for mammalian proteins such as LBP, CD14, and others are hidden in multilamellar aggregates rather than in cubic aggregates. The change of LPS structure into a multilamellar structure induced by the peptides found here (Fig. 6), which has been shown to be connected with large increases in aggregate sizes (2), is a necessary prerequisite for LPS inactivation. In particular, the conversion of the lipid A aggregate into a multilamellar arrangement already at a [lipid A]/[Pep19-2.5] molar ratio of 7:1 shows that the degree of multilamellarization can be correlated directly with the inhibition property in the biological system. SAXS data with less-efficient peptides show that the lipid A aggregate structures are multilamellarized only at a much higher peptide concentration (data not shown).

A second factor is the high binding affinity of LPS to SALPs, which must be higher than that of the LBP-LPS binding. In Fig. 5, it becomes clear that there is an extremely strong Coulomb interaction between the positive charges of the peptide and the negative charges of LPS connected with an incorporation of the peptides into the LPS bilayer, with a saturation taking place already at a [Pep19-2.5]/[LPS] ratio of 3:10 M/M.

In recent years, LPS-neutralizing antimicrobial peptides have been in the focus of sepsis research (18–22, 28). In most cases, however, the applied excess concentration ratio of peptide to LPS, necessary for sufficiently high LPS inactivation, was in the range of 1,000, or even higher, thus being insufficiently selective for LPS with respect to cytotoxic effects against physiological mammalian cells.

The presented data are clearly indicative of the potential ability of the peptides to act as antiseptic therapeutics. Further experiments are in progress using a mouse model of sepsis caused by bacterial infection, i.e., one based on inoculation of Gram-negative bacterial cells rather than isolated LPS and testing the action of the peptides in the absence and presence of conventional antibiotics. Preliminary results show a significant increase of survival only when peptide Pep19-2.5 is administered in combination with an antibiotic (amikacin) (our unpublished data). Delayed addition of the peptide after LPS administration is also in the focus of our interest. Initial testing with the same model of endotoxemia used in the present report still shows a considerable increase in mouse survival when the peptide Pep19-2.5 was added intraperitoneally after 0.5 to 2 h of the LPS challenge. Furthermore, use of other animal models of sepsis such as cecal ligation and puncture is presently ongoing and give evidence that Pep19-2.5 alone is able to significantly increase mouse survival (our unpublished data). These data will be the focus of a follow-up study.

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