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Biophysical analysis of the interaction of granulysin-derived peptides with enterobacterial endotoxins

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Abstract

To combat infections by Gram-negative bacteria, it is not only necessary to kill the bacteria but also to neutralize pathogenicity factors such as endotoxin (lipopolysaccharide, LPS). The development of antimicrobial peptides based on mammalian endotoxin-binding proteins is a promising tool in the fight against bacterial infections, and septic shock syndrome. Here, synthetic peptides derived from granulysin (Gra-pep) were investigated in microbiological and biophysical assays to understand their interaction with LPS. We analyzed the influence of the binding of Gra-pep on (1) the acyl chain melting of the hydrophobic moiety of LPS, lipid A, by Fourier-transform spectroscopy, (2) the aggregate structure of LPS by small-angle X-ray scattering and cryo-transmission electron microscopy, and 3) the enthalpy change by isothermal titration calorimetry. In addition, the influence of Gra-pep on the incorporation of LPS and LPS-LBP (lipopolysaccharide-binding protein) complexes into negatively charged liposomes was monitored. Our findings demonstrate a characteristic change in the aggregate structure of LPS into multilamellar stacks in the presence of Gra-pep, but little or no change of acyl chain fluidity. Neutralization of LPS by Gra-pep is not due to a scavenging effect in solution, but rather proceeds after incorporation into target membranes, suggesting a requisite membrane-bound step.

Keywords: Endotoxin; Lipopolysaccharide; Antimicrobial peptide; Sepsis; Granulysin

1. Introduction

Gram-negative bacteria such as *Escherichia*, *Salmonella*, *Yersinia*, and *Vibrio* are responsible for a multitude of infections. In particular, they are inducers of septic shock syndrome that is lethal in more than 50% of cases [1]. It is well accepted that lipopolysaccharide (LPS), the main amphiphilic compound located in the outer leaflet of the outer membrane, is the major pathogenicity factor of Gram-negative bacteria [2]. When LPS is released from bacteria due to cell division or cell death, it may interact with various target cells to induce tumor necrosis factor-á (TNF-α) and interleukins [3]. At low LPS concentrations, these interactions may be beneficial; however, high LPS concentrations activate a cascade of events that can result in multi-organ failure and septic shock for which there is no effective therapy.

The use of antimicrobial peptides based on endotoxin-binding structures of mammalian defense proteins [4–6] represents a new therapeutic approach to septic shock. Granulysin is a cytolytic protein found in the granules of human cytotoxic T lymphocytes and natural killer cells [7, 8]. The crystal structure of granulysin consists of a five-helix bundle suggesting a potential mechanism of action whereby the positive charges of granulysin orient the molecule towards the negatively charged surface of target cells lysing their membrane [9]. Synthetic peptides corresponding to the linear sequence of granulysin kill Gram-positive and Gram-negative bacteria [10]. Furthermore, NK-lysin, the porcine homolog of granulysin, inhibits LPS-induced cytokine production [11], as does a fragment of NK-lysin [12].

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An ideal antibiotic should both kill bacteria and neutralize the endotoxins subsequently released. In this study, we performed systematic microbiological, physico-chemical and biophysical studies on the interaction of bacterial endotoxins with four granulysin derivatives (Gra-pep). LPS from *Salmonella minnesota* Re and Ra, strains R595 and R60, respectively, were used as endotoxins. We monitored the gel to liquid crystalline phase transition behavior of the acyl chains of LPS within its lipid A moiety, the aggregate structure of LPS, their morphology, the intercalation of the peptides into phosphatidylserine (PS) target membranes in the presence of LPS and lipopolysaccharide-binding protein (LBP), and the influence of the peptides on the LPS-induced cytokine production in human mononuclear cells. Our results suggest that neutralization of LPS by Gra-pep involves association of the complex with immune cell membranes.

2. Materials and methods

2.1. Bacterial strains and growth

The bacterial strains used in the study were wild-type or LPS mutant strains of *Salmonella enterica* (serovar Minnesota) with smooth-form LPS (S form; wild-type), strain R60 with rough LPS (LPS Ra), and strain R595 deep rough LPS (LPS Re). For antibacterial assay, bacteria were grown overnight in N-minimal medium (10.46 g of Bis-Tris, 6.05 g of Tris Base, 0.087 g of K_2SO_4, 0.136 g of KH_2PO_4, 0.372 g of KCl, 1.0 g of $(NH_4)_2SO_4$, 0.1% casamino acids, 38 mM glycerol, 1.0 l distilled water) supplemented with 10 mM $MgCl_2$ (pH 7.4) at 37 °C [13]. Cells were subsequently washed twice with N-minimal medium, inoculated in N-minimal medium + 10 μM $MgCl_2$ (pH 7.4) and grown to mid-exponential phase. Cells were washed twice with N-minimal medium + 10 μM $MgCl_2$ (pH 7.4) before assay.

2.2. Synthesis of granulysin peptides

Peptides were synthesized using F-moc chemistry on an Applied Biosystems (Foster City, CA) automatic peptide synthesizer and purified to >95% homogeneity by reverse-phase HPLC. Peptide composition was confirmed by mass spectrometry and amino acid analysis. Peptide stock solutions (10 mM) were prepared in DMSO and diluted into assay medium at 0.078–10 μM.

The sequences of the Gra-pep are shown in Table 1. These peptides are based on helix 3 and helix 4 of granulysin. This region was previously determined to be important for antibacterial activity [10]. To increase resistance to proteases, all Gra-pep were synthesized with D-amino acids.

Table 1
Amino acid sequences of granulysin-derived peptides (Gra-pep)

Peptide	Sequence	Net positive charge	Hydrophobic residues	Prolines
Helix3/ Helix4	RDVCRNFMRRYQSRVIQGLV	5	8	0
G12.34	**r**r**v**s**rr**fmrry**r**s**r**i**rr**lv	11	6	0
G12.35	rrvsrrfmrryrsr**p**rrlv	11	5	1
G12.21	rrvsr**p**mrryrsr**p**rrlv	11	4	2
G12.25	rrvsr**ppp**rryrsr**p**rrlv	10	3	4

Uppercase letter represent L-amino acids and lowercase letter represent D-amino acids.
Amino acids substitution of granulysin Helix 3 and Helix 4 regions are indicated in bold.
Helix 3: RDVCRNFMRR; Helix 4: YQSRVIQGLV.

2.3. Assay for antibacterial activity

Microtiter plate assay: each peptide was diluted to 20 μM in assay medium and 100 μl of this solution were added to the first well of a 96-well microtiter plate. For twofold serial dilution, 50 μl from each well were transferred to the next well, which contained 50 μl of assay medium. Subsequently, 50 μl of bacteria at 2×10^5 CFU/ml (1×10^4 cells) were added to each well. The plates were incubated for 90 min at 37 °C with constant shaking. After 90 min, 100 μl of 2× LB were added to each well, and the plates were further incubated with shaking overnight at 37 °C. Bacterial growth was monitored by measuring the absorbance at 600 nm in a microtiter plate reader (Molecular Devices, California, USA). The MIC (minimal inhibitory concentration) is defined as the lowest peptide concentration at which no bacterial growth was measurable after overnight incubation.

Colony forming unit (CFU) assay: bacteria and Gra-pep were added to microtiter wells as described above and, after a 90 min incubation at 37 °C, bacteria were serially diluted in PBS and plated on LB agar. The plates were incubated overnight at 37 °C, and bacterial colonies were enumerated the following day by automatic colony counter (aCOLyte colony counter, UK). The MBC (minimal bactericidal concentration) is defined as the lowest peptide concentration at which less than 1% of the input bacteria survived.

To test whether pre-incubation of Gra-pep with LPS affects antibacterial activity, 50 μl of different molar ratio of LPS (S-form or LPS Re) and peptide G12.21 were incubated in N-minimal medium + 10 μM $MgCl_2$ (pH 7.4) in microtiter wells at room temperature with shaking for 30 min. Then, 50 μl of bacteria (wild-type or strain R595) at 2×10^5 CFU/ml were added. After an additional 90-min incubation at 37 °C with constant shaking, aliquots were removed, diluted, and plated for CFU assay. The percent survival was determined as CFU (Gra-pep:LPS)/CFU (medium) × 100.

2.4. Lipopolysaccharides and lipid:peptide samples

Lipopolysaccharides from the rough mutant Re and Ra *S. minnesota* (strains R595 and R60, respectively) were extracted by the phenol/chloroform/petrol ether method [14], and S-form LPS was extracted according to the phenol:water procedure [15]. Briefly, an overnight culture of bacteria grown at 37° was purified and lyophilized. The schematic structures of LPS presented in Fig. 1 shows that the two mutant LPS molecules differ essentially in the length of the carbohydrate chain and in the number of negative charges. Lipopolysaccharide-binding protein (LBP) was a kind gift of Russ L. Dedrick (XOMA Co, Berkeley, CA, USA) and was stored at −70 °C at 1 mg/mL stock solution in 10 mM HEPES (150 mM NaCl, 0.002% (v/v) Tween 80, 0.1% F68, pH 7.5). Bovine brain *3-sn*-phosphatidylserine (PS) was from Sigma.

All lipid samples were prepared as aqueous suspensions in 20 mM HEPES (pH 7.0). Briefly, the lipids were suspended directly in buffer and were temperature-cycled 3 times between 5 and 70 °C, interrupted by intensive vortex, and then stored 2 h at 4 °C before measurement. To guarantee physiological conditions, the water content of the samples was ~95%. For preparations of phosphatidylserine liposomes, phosphatidylserine was first solubilized in chloroform, and the solvent was evaporated under a stream of nitrogen. Then, the lipid was resuspended in the appropriate volume of 20 mM HEPES, and treated as described above (temperature-cycling). The resulting liposomes are large and multilamellar as shown by electron microscopy (kindly performed by H. Kühl, Division of Pathology, Forschungszentrum Borstel).

2.5. Cytokine assay

Human mononuclear cells (MNC): heparinized (20 IU/ml) blood obtained from healthy donors was mixed with an equal volume of Hank's balanced solution, layered over Ficoll, and centrifuged for 40 min (21 °C, 500×*g*). The interphase layer of MNC was collected and washed twice in Hank's medium and then resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 4% heat-inactivated human serum type AB from healthy donors. MNC (200 μl /well; 5×10^6 cells/ml) were transferred into 96-well culture plates. Twenty microliters of a mixture containing LPS Ra (100 ng/ml or 10 ng/ml) and Gra-pep (10:1 wt.% excess) was added to each well. Supernatants were harvested after 4 h incubation at