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Galleria mellonella native and analogue peptides Gm1 and ΔGm1. II) Anti-bacterial and anti-endotoxic effects

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ABSTRACT

Antimicrobial peptides (AMPs) are important components of the innate immune system of animals, plants, fungi and bacteria and are recently under discussion as promising alternatives to conventional antibiotics. We have investigated two cecropin-like synthetic peptides, Gm1, which corresponds to the natural overall uncharged *Galleria mellonella* native peptide and ΔGm1, a modified overall positively charged Gm1 variant. We have analysed these peptides for their potential to inhibit the endotoxin-induced secretion of tumour necrosis factor-α (TNF-α) from human mononuclear cells. Furthermore, in a conventional microbiological assay, the ability of these peptides to inhibit the growth of the rough mutant bacteria *Salmonella enterica* Minnesota R60 and the polymyxin B-resistant *Proteus mirabilis* R45 was investigated and atomic force microscopy (AFM) measurements were performed to characterize the morphology of the bacteria treated by the two peptides. We have also studied their cytotoxic properties in a haemolysis assay to clarify potential toxic effects.
Our data revealed for both peptides minor anti-inflammatory (anti-endotoxin) activity, but demonstrated antimicrobial activity with differences depending on the endotoxin composition of the respective bacteria. In accordance with the antimicrobial assay, AFM data revealed a stronger morphology change of the R45 bacteria than for the R60. Furthermore, Gm1 had a stronger effect on the bacteria than ΔGm1, leading to a different morphology regarding indentations and coalescing of bacterial structures. The findings verify the biophysical measurements with the peptides on model systems. Both peptides lack any haemolytic activity up to an amount of 100 μg/ml, making them suitable as new anti-infective agents.

1. Introduction

Infectious diseases represent a major challenge in medical science, exacerbated by the increase of resistant microbes. It demands immediate measures, such as a more stratified treatment of patients by the existing drugs and the development of new antibiotics [1,2]. Independent of this fact, a main issue is that conventional antibiotics can cause cell lysis during bacterial killing, leading to a release of endotoxins (lipopolysaccharides, LPS) from the outer membrane of Gram-negative bacteria. This release can lead to a worsening of the patients' health status due to an increase in inflammation [3]. Released LPS is one of the most potent activators of the human immune system and may cause sepsis, severe sepsis and septic shock [4]. Therefore, one main demand is the development of drugs which are able to neutralize bacterial endotoxins [5]. One approach may be the use of antimicrobial peptides (AMPs), which may combine the effects of microbial killing and endotoxin neutralization. For example, some insect species can express an individual set of AMPs in response to invading microorganisms, which may also exert anti-endotoxin activity [6,7]. Here, we focus on host defence AMPs from insects [8,9], which are thought to act by a rapid destruction of bacterial membranes [10,11]. A particular species *Galleria mellonella* (*G. mellonella*) concurrently produces an impressive array of at least 18 known or putative AMPs from 10 families to defend against invading microbes, which may also exert anti-endotoxin activity [12,13]. Gm1, a native peptide with no net charge obtained from *G. mellonella*, is the peptide with the broadest activity spectrum, which has proven activity

Abbreviations: AMPs, antimicrobial peptides; Gm cecropin D-like peptide, *Galleria mellonella*; Gm1, native peptide; AFM, atomic force microscopy; PMB, polymyxin B; LPS, lipopolysaccharides; PCP, phenol/chloroform/petrol ether; MNC, mononuclear cells; TNF-α, tumour necrosis factor-α; MIC, minimum inhibitory concentration; BSA, bovine serum albumin

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against different kinds of Gram-positive bacteria and *Escherichia coli* (*E. coli*) D31 as well as filamentous fungi [14]. We have taken Gm1 as a promising candidate peptide for elucidating its ability to neutralize the toxic properties of LPS components of the outer membrane of Gram-negative bacteria [15], to kill bacteria and to analyse the killing mechanism. As a modified structure the compound ΔGm1 was investigated, which is overall positively charged due to an exchange of 5 amino acids to cationic ones. In this second part of two manuscripts, we focus on the elucidation of the biologic activity of the two peptides. Therefore we investigated the microbial susceptibility in a conventional minimal inhibitory concentration assay, performed a comprehensive study of the morphology of the bacteria in the presence of the peptides by atomic force microscopy and tested their LPS-neutralizing activity in an in vitro assay of LPS cell stimulation by analysing the peptide induced inhibition of the secretion of TNF-α.

2. Materials and methods

2.1. LPS and peptides

LPS Ra (R60) and Re (R595) from *Salmonella enterica* Minnesota and R45 from polymyxin B-resistant *Proteus mirabilis* were extracted from the bacteria by phenol/chloroform/petrol ether (PCP) method [16], purified and the composition was analysed using Maldi-TOF mass spectrometry. The chemical structures of the *S. enterica* LPS are drawn schematically in Fig. 1.

The purification and characterization of the native peptide Gm1 (ENFFKE**I**ER**A** GQRIRDAIIS**AA**P**A**VETLAQAQKIIKGGD) were described for the first time in previous publications [14,17]. The peptides Gm1 and ΔGm1 (ENFFKE**K**ER**K**GQRIRDAIIS **RR**P**R**VETLAQAQKIIKGGD) were synthesized without an amidated C-terminus by the Fmoc solid-phase synthesis technique with an automatic peptide synthesizer (433 A Applied Biosystems Synjthesizer).

2.2. Determination of the TNF-α expression after stimulation of human cells with endotoxin

Mononuclear cells (MNC) were isolated from heparinized peripheral blood taken from healthy donors. Therefore 100 ml of whole blood was mixed in a 1:1 ratio with Hanks Balanced Salt Solution (Gibco, Invitrogen, California, USA). The mixture was subdivided in 33 ml portions and each was added to 10 ml Biocoll separation solution (Merck, Darmstadt, Germany). The density gradient centrifugation was performed at 4 °C for 30 min at 600 ×*g*. The supernatant was removed and the turbid MNC layer was transferred in 2 50 ml plastic tubes. To the supernatants Hanks solution was added until a final volume of 50 ml was reached. The tubes were centrifuged at 4 °C with 400 ×*g* for 10 min. The supernatants were discarded, the cell pellets resuspended in 5 ml Hanks solution each and combined in 1 50 ml tube. 2

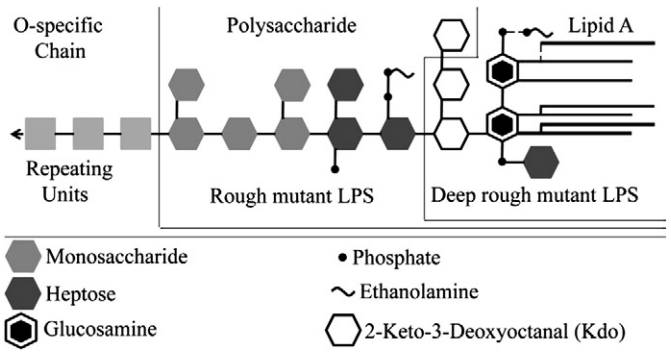

Fig. 1. Schematic representation of the rough type lipopolysaccharides R60 and R595 from *Salmonella enterica*.

further washing cycles with Hanks solution and 1 cycle with RPMI medium 1640 (Biochrom AG, Berlin, Germany) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin were performed before the cells were resuspended in 10 ml RPMI medium. The cell number was equilibrated at 5×10^6 MNC/ml with RPMI medium. For stimulation, 200 μl/well MNC was transferred into 96-well culture plates. LPS-stimuli was serially diluted in RPMI 1640 without antibiotics and added to the cultures at 20 μl per well to obtain a final concentration of 1 ng/ml (0.23 nM). Afterwards the peptides were added to the cultures at final concentrations of 100 ng/ml (Gm1 23.5 nM; ΔGm1 21.8 nM). The cultures were incubated for 4 h at 37 °C under 5% CO_2. Cell-free supernatants were collected after centrifugation of the culture plates for 10 min at 400 ×*g* and stored at −20 °C until determination of the cytokine content. Immunological determination of TNF-α was performed in duplicate as described in the protocol (OptEIA; BD, Heidelberg, Germany).

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Data of 4 individual healthy donors were compared by the Student two-tailed *t* test (*$p < 0.05$).

2.3. Minimal inhibitory concentration

The determination of the minimal inhibitory concentration (MIC) was performed in compliance with the method described by the European Committee for Antimicrobial Susceptibility Testing [18]. Briefly, the bacteria were grown in a preculture overnight at 37 °C gently shaken. 1 ml of the culture was transferred to 49 ml lysogeny broth medium (LB; Merck, Darmstadt, Germany) until an OD_{600} of 0.7 was reached. The bacteria are adjusted to 1×10^6 bacteria/ml suspension and 10 μl was transferred to a 96 well plate loaded with 90 μl of the peptide solutions in 20 mM HEPES pH 7.4. The plate was incubated overnight at 37 °C under constant gentle shaking, subsequently the OD_{620} was measured in a Tecan Reader infiniteM200Pro. Three independent experiments were performed and the mean value was calculated. The MIC (minimal inhibitory concentration) was defined as the lowest peptide concentration at which no bacterial growth was measurable. Experiments were repeated three times and the shown results represent the MICs with and error of one dilution step.

2.4. Atomic force microscopy (AFM)

The alterations in the bacterial cell surface caused by Gm1 and ΔGm1 were investigated using the AFM MFP-3D (Asylum Research, Santa Barbara, CA, U.S.A.). Briefly, exponential-phase bacterial cultures (5×10^8 CFU/ml of *P. mirabilis* R45 and *S. enterica* R60 in 100 μl of LB medium) were incubated without additives, BSA (100 μg/mL) and melittin (10 μM) as negative and positive controls or peptides (10 μg/ml), respectively. The additives plus bacteria were incubated at 37 °C for 30 min. Then 50 μl of bacterial suspension was placed on mica, excess of liquid was removed and the bacteria were air-dried at room temperature for 24 h. Subsequently the samples were rinsed with 3 ml di-water to remove salt crystals, dried again and imaged with a CSG 11 cantilever ($k = 0.1$ N/m; NT-MDT) in contact mode, using line-frequencies of 1.0 Hz and 512 points per lines. Further image processing (flattening and plane fitting) was done with the MFP-3D software under IGOR Pro (Lake Oswego, OR, U.S.A.). Images shown here are representative of the respective samples. All samples were repeated three times in independent experiments.

2.5. Haemolytic activity of peptides

Freshly isolated human erythrocytes were obtained from citrated human blood of 3 individual healthy donors by centrifugation (1500 ×*g*, 10 min), washed three times with isotonic PBS buffer, pH 7.4 at 37 °C and suspended in the same buffer. The activity of the peptides to lyse human erythrocytes was determined by dilutions