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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.

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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

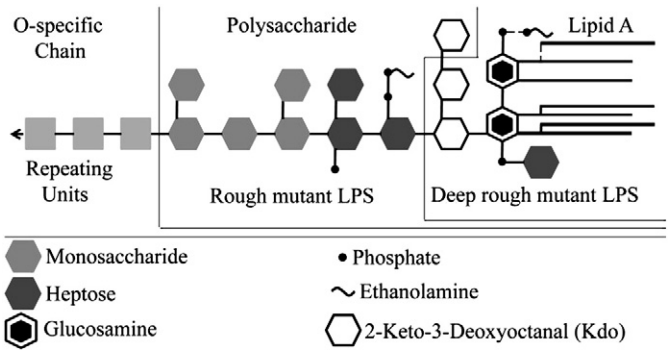

Fig. 1. Schematic representation of the rough type lipopolysaccharides R60 and R595 from *Salmonella enterica*.

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

prepared in duplicate in a round bottom microtiterplate. For that, 20 μl of erythrocytes (5×10^8 cells/ml) was incubated with 80 μl of a peptide sample at 37 °C for 30 min in a humidified box. The haemolytic activity after incubation was measured by transferring the supernatants into another empty microtiter plate. The optical density was measured in a plate reader at 405 nm. Haemolytic activity was expressed as percentage of released haemoglobin with respect to water controls (100% release) or controls processed without peptides (0% release). Shown results are averages of three independent experiments.

3. Results

3.1. Inhibition of the production of cytokines from human mononuclear cells

The LPS-induced production of TNF-α from human mononuclear cells was determined and the potential inhibition by the addition of Gm1 and ΔGm1 at a LPS/peptide ratio of 1:100 wt.% (Fig. 2). Both peptides caused a minor inhibition of the LPS-induced TNF-α secretion, with the peptide Gm1 showing a statistically significant and slightly higher inhibition of LPS R60 than ΔGm1. This suggests that Gm1 and ΔGm1 can block the action of free endotoxin partially, however, the level of the inhibition is moderate.

3.2. Antibacterial activity

In order to compare the antibacterial activity of Gm1 and ΔGm1, we used four different rough mutant enterobacteria. *S. enterica* Minn. R60, R595, *E. coli* Re and *P. mirabilis* R45 representing different negatively charged LPS backbones with the longest carbohydrate chain in the R60 and the deep rough mutants R595, Re and R45, which differ mainly in substitutions of aminoarabinose, with the highest negative charge for the Re, then the R595 and with the lowest overall charge for the R45 (Table 1). Gm1 outperforms ΔGm1 in all cases, which seems to be surprising due to the fact that the latter has 5 positive charges assumed to bind better to the negatively charged LPS backbones than the overall uncharged compound Gm1.

3.3. Morphological changes of bacterial surfaces after Gm1 and ΔGm1 treatment: imaging by AFM

We focused our AFM analyses on the *P. mirabilis* R45 strain in order to see the morphological changes of a polymyxin B-resistant strain induced by the action of peptides [19,20]. This strain displays a chemotype of LPS that has a non-stoichiometric substitution of an amino-arabinose, which leads to a reduction of the negative net charge as compared to LPS R595 or LPS R60 (Fig. 1) [21,22]. For this we used BSA and mellitin as a negative and positive control respectively. The concentration of melittin used was reported in a previous work to be close at the MIC value found for this strain in presence of 20 mM Hepes, pH 7.4, and 150 mM NaCl, supplemented with 10% LB medium [23]. This concentration allows us to compare the MIC values of the peptides studied here. The AFM data, obtained at Gm1 and ΔGm1 concentrations of 10 μM, revealed subtle changes caused on the bacterial cell surface with high-resolution and low-distortion images and it has been employed for elucidating the interaction of the peptide NK-2 with *E. coli* and *P. mirabilis* R45 [23]. Effects of Gm1 and ΔGm1 treatment on the morphology of the bacteria assessed by AFM are shown in Figs. 3 and 4. A normal morphology of the membrane surfaces and a good definition of borders between bacteria and also lysed cells resulted from the normal process of growth (Figs. 3 and 4A, B). *P. mirabilis* R45 exhibits a tendency to form clusters of cells itself, while *S. enterica* R60 is distributed rather individually.

Gm1 shows a drastic impact on the structural integrity of both bacteria (Figs. 3 and 4D). It led to a different morphology, indentations and a loss of cytoplasm was observed. However, the most considerable observation was the apparent fusion of membranes, which led to a blurring of the borders between single bacteria. The picture represented by ΔGm1 not only differs from Gm1, but an increased attachment of the cells to each other was also observed (Fig. 4E). However, the treatment of the two peptides led to a different effect on the bacteria, then the strong cell disrupting peptide melittin, whereby for the former the effect can be described as predominantly clotting and membrane fusing and for the latter as cell lysing.

Table 1
Minimal inhibitory concentration (MIC) of Gm1 and ΔGm1 to different enterobacteriaceae.

Bacteria	Gm1 (μM)	ΔGm1 (μM)
S. enterica R60	40	>160
S. enterica R595	<1.25	20
P. mirabilis R45	5	>160
E. coli Re	<2.5	20

3.4. Haemolysis of Gm1 and ΔGm1

As measure for the results reflects the assumed differences in the interaction mechanisms and corroborate our biophysical findings, that Gm1 is a stronger inducer of bacterial destructions, which leads to dissolution of the single bacteria. Possible cytotoxic activity of the peptides, Gm1 and ΔGm1 were analysed in a haemolysis assay to test the ability to lyse erythrocytes. The highest concentration of 199 μg/ml of each tested peptide does not show any sign of cytotoxicity, whereas synthetic bee venom melittin, as well-known positive control, exhibits already at rather low concentrations of essential haemolytic activity (Fig. 5).

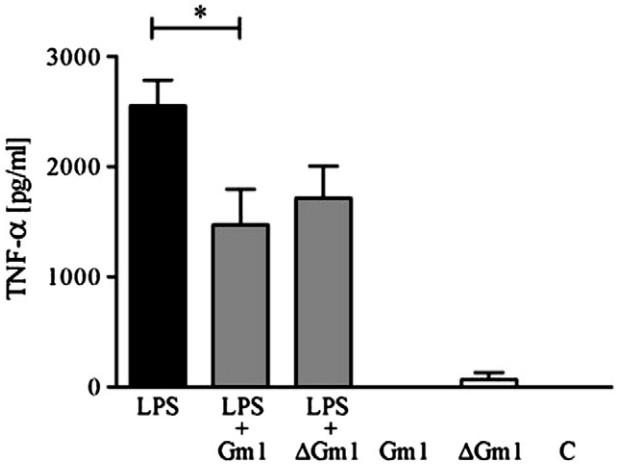

Fig. 2. LPS R60 (1 ng)-induced TNF-α expression from human MNC in the absence and presence of peptide Gm1 and ΔGm1 (100 ng) was determined. 4 independent experiments were performed and compared by the use of the Stundent *t* test (*$p < 0.05$).

4. Discussion

In the present study, we investigated the biological activity of two peptides, the natural peptide, Gm1 and the overall-positively charged variant, ΔGm1, whereby the focus was on the interaction with Gram-negative bacteria.

The biophysical analysis of the interaction of the peptides with relevant lipids from the outer membrane of Gram-negative bacteria, i.e., negatively charged dimyristoylphosphatidylglycerol and lipopolysaccharides (LPS) was performed in a preceding study. We showed considerable differences between the two peptides, Gm1 led to a fluidization of the acyl chains of the lipids in the macromolecular structures, whereas ΔGm1 had a rigidifying effect on these. Furthermore, the binding characteristics of the peptide to the bacterial LPS revealed differences. While both peptides provided similar values for the Gibbs free energy

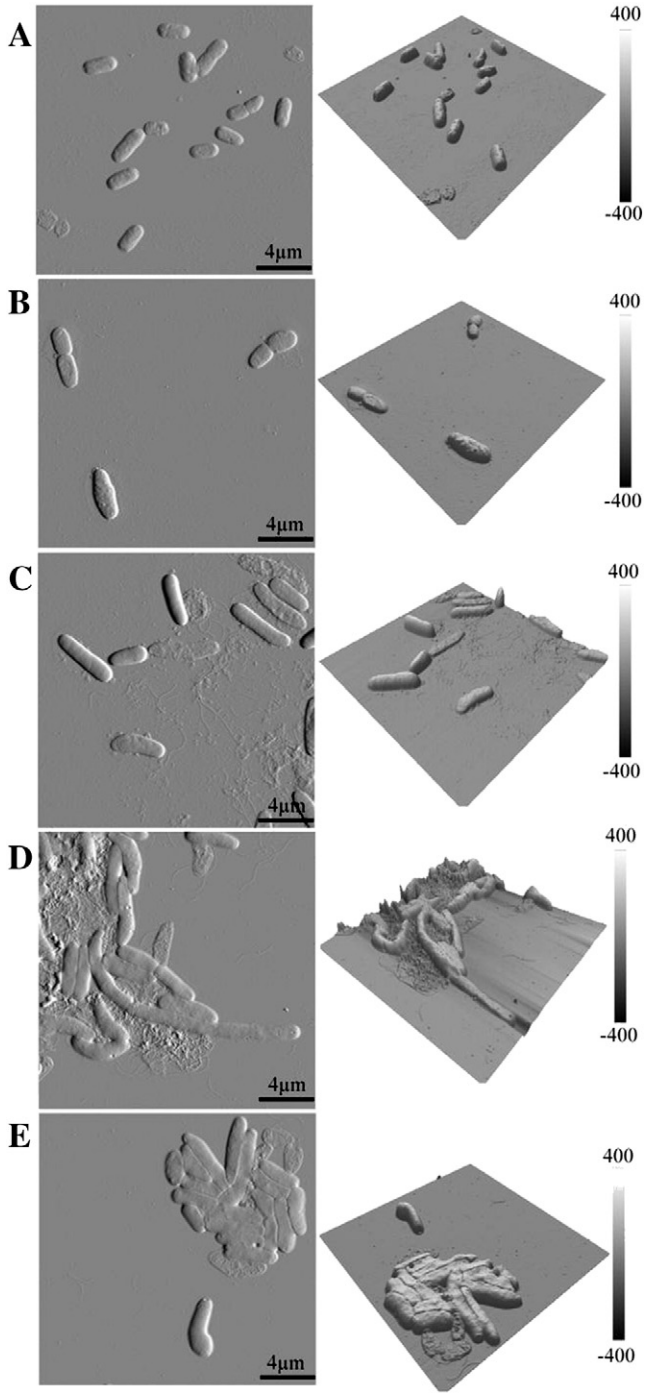

Fig. 3. AFM images of alterations in the *S. enterica* R60 cell surface. The bacteria were incubated without (control) (A) or in the presence of BSA (100 μg/ml) (B) or melittin (10 μM) (C). *S. enterica* R60 cell surface after incubation with Gm1 and ΔGm1 (10 μM) are shown in (E) and (D) respectively. The left panels show deflection images and the right panels are 3D reconstructions. The images were performed in air in contact mode.

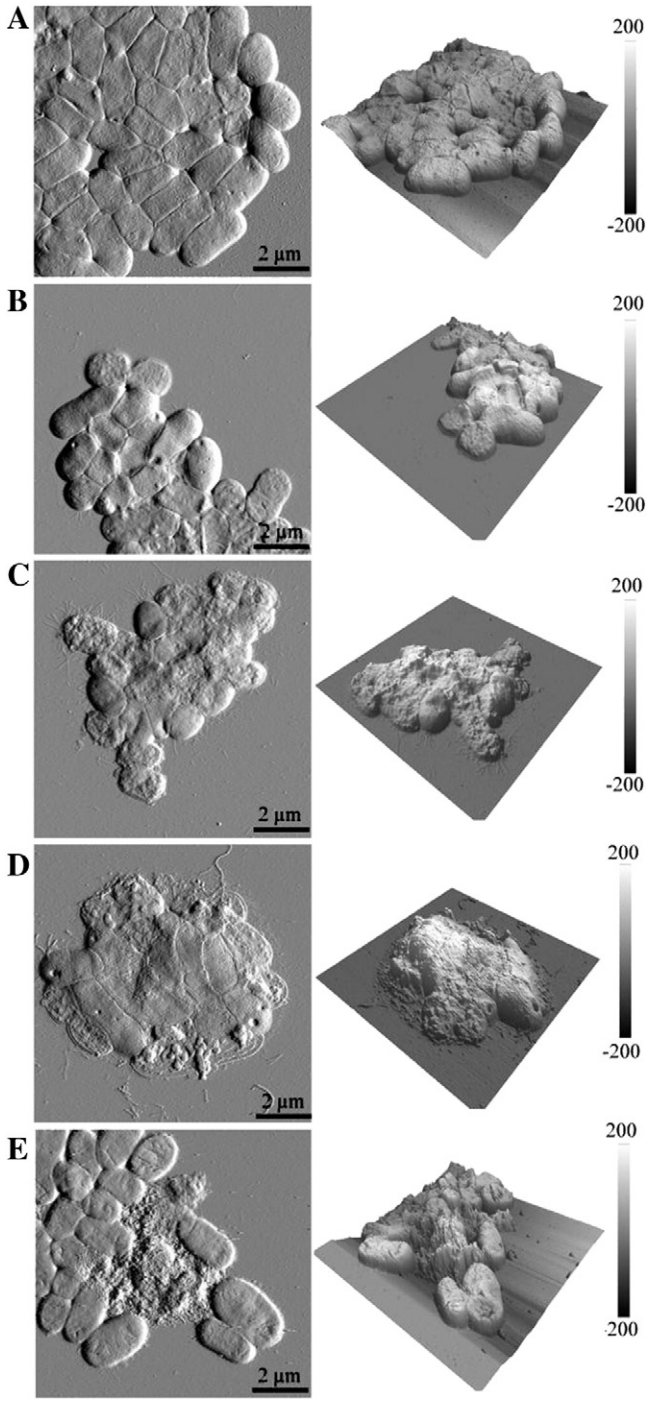

Fig. 4. AFM images of alterations in the *P. mirabilis* R45 cell surface. The bacteria were incubated without (control) (A) or in the presence of BSA (100 μg/ml) (B) or mellitin (10 μM) (C). *P. mirabilis* R45 cell surface after incubation with Gm1 and ΔGm1 (10 μM) are shown in (D) and (E) respectively. The left panels show deflection images and the right panels are 3D reconstructions. The images were performed in air in contact mode.

(ΔG), they differed in the strength of the exothermic reaction (ΔH) and the entropic effect (−TΔS). Surprisingly, the enthalpy of the LPS interaction was stronger for Gm1, while the entropy effect seems to be more pronounced for ΔGm1. The impact of these differences is reflected by the biologic activity regarding the neutralization of LPS, the antimicrobial activity, the haemolytic activity and the direct observation of bacteria by atomic force microscopy.

The LPS-neutralizing effect observed for both peptides can be described as rather weak compared to known anti-LPS peptides [24,25]. A statistically significant effect could be shown for the neutralization of Gm1 from LPS, but the overall difference between both peptides appears to be small. However, more prominent is the antimicrobial activity of the peptide, Gm1 demonstrated an effect against *S. enterica* and *E. coli*, with a better activity against the deep-rough mutant than to the rough mutant, as expected. Remarkably, antimicrobial activity is better pronounced against the polymyxin-resistant *P. mirabilis*. An explanation could be that the Gm1 peptide has no overall net charge whereas polymyxin B is positively charged, like the large majority of

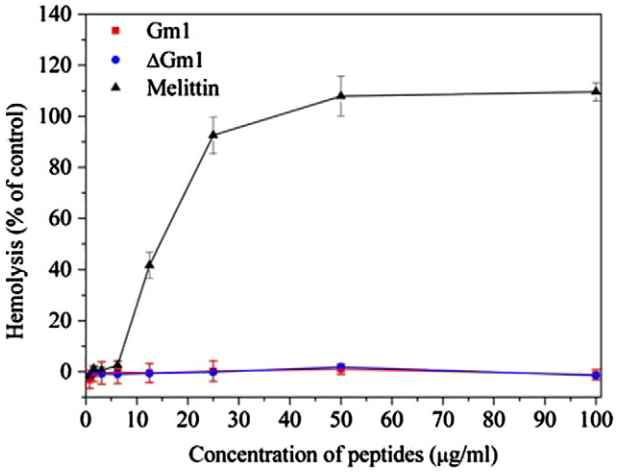

Fig. 5. Haemolytic activity of samples Gm1 and ΔGm1 in comparison to melittin, an extensively studied haemolytic peptide .

antimicrobial peptides [26]. The resistance of this strain consists of the addition of aminoarabinose to the negatively charged LPS molecule to reduce the net charge and therefore the binding strength of the antimicrobial peptide. The antimicrobial activity of ΔGm1 turned out to be negligible.

Extended information about the antimicrobial activity is gained by the morphological changes, monitored by AFM. For the control peptide melittin, with a known strong antimicrobial activity [23], membrane disruption and blebs could be observed frequently associated with a spreading out of the cytosolic contents. Similar observations were made for other AMPs with membrane attacking actions [23,27,28]. The picture for Gm1 and ΔGm1 is markedly different (Figs. 3 and 4). Both peptides demonstrated a clotting effect on the *S. enterica* rough type bacteria, with a slightly pronounced consequence for Gm1 (Fig. 3 D, E). *P. mirabilis* may reveal similar clotting effects, but this is hard to identify, because of the regular tendency of the bacteria to stack together. Nonetheless the AFM images reveal differences in the agglomeration, which might be discussed as membrane fusion effects. This is expressed by a complete dissolution of the single bacterial cells leading to one unstructured assembly, in which the single bacterial cells lose their individual shapes (Figs. 3 and 4D,E). The manner of bacterial killing, accompanied with a formation of cell clusters, could be an interesting observation with regard to the release of free toxins from the cell surface. The aspect of endotoxin release during antibiotic treatment is a frequently observed phenomenon [3], which could be reduced by the detected effects. Thus, AFM represents a direct non-optical technique, which is able to monitor sensitively already slight changes in the morphology of bacterial cells.

The next step could be to study the action of the peptides Gm1 and ΔGm1 also in an animal model of infection. It is important to note, however, that the use of peptides systemically, in particular against bacterial sepsis, is hampered by a possible cytotoxicity of peptides, as described for others [29-31]. As a good prevision of probably no cytotoxicity of the analysed peptides could be the hint that in our in vitro experiments no significant haemolysis up to 100 μg/ml per used peptide could be detected. Therefore the peptides Gm1 and ΔGm1 seem to be suitable for medical systemic use.

Acknowledgements

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