INTERACTION OF BASIC AMPHIPHILIC POLYPEPTIDE ANTIMICROBIALS, GRAMICIDIN S, TYROCINID AND EFRAPEPTIN, WITH ENDOTOXIC LIPID A

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Summary:
Gramicidin S, Tyrocind and Efrapeptin, basic hydrophobic peptide antimicrobials, bind lipid A with apparent Kₐₘ of 1.68 µM, 2.65 µM and 4.92 µM respectively, and inhibit lipid A activity in the Limulus amoebocyte lysate gelation and splenocyte proliferation assays. The results suggest that endotoxin binding properties may be a general property of cationic amphiphilic peptides.

Introduction:
Lipopolysaccharides (LPS) or endotoxins, amphiphilic macromolecular constituents of gram negative bacterial outer membranes, play a central role in the pathogenesis of endotoxic shock and the sepsis syndrome [14]. The lipid component of LPS termed lipid A constitutes the toxic principle of endotoxins [5]. We have attempted to examine the basis of interactions with lipid A, of compounds with endotoxin-antagonistic properties with a view to identifying structural features which ascribe endotoxin-neutralizing attributes to these compounds. We have shown that melitin, a 26-residue amphiphilic, cationic polypeptide component of bee venom binds lipid A and attenuates its biological activity [1]. The interaction of melitin with lipid A was characterized using the intrinsic fluorescence of the peptide [1]. We subsequently developed a fluorescent displacement probe method to characterize the binding of nonfluorescent molecules to lipid A and analyzed the interaction of polymyxin B, a cyclic
decapptide antibiotic, with lipid A [2]. From these earlier studies, we surmised that basicity and hydrophobicity may be common structural features of endotoxin-antagonists. We have now tested this hypothesis by examining the lipid A binding characteristics of three basic and hydrophobic polypeptides of diverse sequences and secondary structures: gramicidin S, a cyclic decapptide antibiotic with a rigid β-pleated structure from Bacillus brevis [10], tyrocidin, a closely related decapptide also from B. brevis [12], and efrapeptin, a linear, α-aminoisobutyric acid-rich peptide from Tolypocladium niveum with an unusual basic moiety attached to the carboxyl terminus [6].

Materials and Methods:

Gramicidin S, and lipid A from Salmonella minnesota Re 595 were from Sigma Chemicals (St. Louis, MO) and tyrocidin hydrochloride (a mixture of components A, B, and C) was from Serva (Heidelberg, Germany). The efrapeptins were isolated from Tolypocladium niveum inflatum and characterized as reported earlier [11]. Stock solutions of the peptides were in methanol. Aqueous stock suspension of lipid A (2.5 mg/ml) was prepared by sonication with trace quantities of triethylamine. Dansylcadaverine (DC) was synthesized as described previously [15] and fluorescence experiments employing DC as a displacement probe were performed as described earlier [2]. Briefly, the binding of DC to lipid A results in a blue-shift and intensity enhancement in the emission spectrum of DC. Compounds which bind lipid A competitively displace DC, resulting in quenching of fluorescence. Apparent dissociation constants for peptide-lipid A interactions are computed from the displacement data by the Horovitz-Levitzi method [8]. Comparisons of the DC displacement curves to evaluate relative potencies of the compounds were performed by simultaneous curve-fitting to obtain accurate ED50 values using the ALLFIT program [3]. Limulus amebocyte lysate (LAL) endpoint gelation and splenocyte proliferation assays were performed as described earlier [1].

![Graph](image_url)  
**FIGURE 1:** Displacement of DC bound to lipid A. Gramicidin S: circles; Tyrocidin: triangles; Efrapeptin: squares. ED50 values are 1.67 mM, 2.94 mM, and 6.23 mM, respectively. DC occupancy is calculated as (F-Fo)/(Fmax-Fo) [2]. Fo: fluorescence intensity of probe alone; Fmax: fluorescence intensity of the probe-lipid A mixture at 0 μM peptide concentration.
FIGURE 2. Mitogenic activity of free lipid A and complexed lipid A. Tritiated thymidine incorporation in BALB/c murine splenocytes. A constant peptide/lipid A molar ratio was maintained at different lipid A concentrations. Doses indicated correspond to that of lipid A in the case of free lipid A (solid bars) and peptide-lipid A complexes (vertical stripes). Concentrations of free peptides (horizontal stripes) are identical to that present in corresponding peptide-lipid A mixtures. Error bars represent standard deviations calculated on quadruplicate samples. Also shown are RPMI (zero lipid A and/or peptide concentration) and concanavalin A controls (blank bars).

Table 1

<table>
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<th>100ng</th>
<th>10ng</th>
<th>1ng</th>
<th>100pg</th>
<th>10pg</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
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<td>-</td>
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<td>-</td>
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<tr>
<td>LA + Tyrocidin</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>LA + Efrapeptin</td>
<td>+</td>
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Results and Discussion:

The antimicrobial peptides bind lipid A, resulting in the displacement of bound DC (fig.1). Unlike polymyxin B, these peptides do not elicit a biphasic displacement of the probe [2], suggesting cooperative behaviour. Apparent Kₘs obtained from the Horovitz-Levitzki plots (data not shown) for gramicidin S, tyrocidin and efrapeptin were 1.68 μM, 2.65 μM and 4.92 μM, respectively.
In the LAL assay, concentrations of 100 pg/ml and higher of lipid A produce LAL gelation (table 1). Mixtures of lipid A and 10-molar excesses of gramicidin and tyrocidin do not elicit gelation even at 100ng/ml of lipid A, while efrapeptin-lipid A mixtures produce gelation only at the highest concentration of lipid A tested (100ng/ml). The addition of excess lipid A (50 µl of 5µg/ml) resulted in gelation showing that the peptides did not have intrinsic LAL-inhibitory activity. In experiments performed with drug/lipid A molar ratios of 2:1, gelation was observed at the highest concentration of lipid A used (100ng/ml) in the case of complexes of lipid A with gramicidin S and tyrocidin, while the efrapeptin-lipid A complex induced gelation at 100ng/ml and 10ng/ml of lipid A, reflecting that a greater fraction of lipid A was unbound at lower concentrations of the peptides.

The splenocyte proliferation assays (fig. 2) were performed with the indicated concentrations of lipid A preincubated with 10-molar excesses of the peptides; peptides alone at corresponding concentrations served as controls. At 0.5µg lipid A concentration, gramicidin and tyrocidin attenuate [3H]thymidine incorporation, while the peptides themselves are without effect. Efrapeptin, however, is toxic at this concentration which precluded comparisons of antagonistic efficacy of the peptides in this assay. These results demonstrate a significant inhibition of the mitogenic activity of lipid A when presented to murine splenocytes as complexes with the peptides.

The above data are indicative of direct interaction of the antimicrobial peptides and consequent attenuation of the activity of lipid A in vitro. Taken together with our earlier work with melittin [1], it appears that neither the primary amino acid sequence, nor the secondary structure seems to be crucial in determining the lipid A-binding properties of peptides. This is consistent with previous studies which have demonstrated that a wide array of cationic and hydrophobic substances bind LPS and lipid A, including the aminoglycoside antibiotics [4,7], cationic dyes [9], and basic proteins [13,16]. This diversity suggests that basicity and hydrophobicity may be both sufficient and necessary for binding to lipid A or LPS.

A detailed characterization of the complexes are necessary for a comprehensive understanding of the structural basis of the interactions. Unfortunately the solubility characteristics of lipid A precluded detailed analyses of the complexes by NMR methods. We are now screening cationic amphiphilic nonpeptide molecules for lipid A-antagonistic properties. Complexes of structurally well-defined small molecules with lipid A may be more amenable to detailed characterization and are to likely provide a basis for establishing quantitative structure-activity relationships of these compounds. The identification of molecules which abrogate the toxicity of lipid A may prove useful in developing therapeutic strategies.
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References:


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