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Characterization of the interactions of a polycationic, amphiphilic, terminally branched oligopeptide with lipid A and lipopolysaccharide from the deep rough mutant of *Salmonella minnesota*

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Summary The lipid A and lipopolysaccharide (LPS) binding and neutralizing activities of a synthetic, polycationic, amphiphilic peptide were studied. The branched peptide, designed as a functional analog of polymyxin B, has a six residue hydrophobic sequence, bearing at its N-terminus a penultimate lysine residue whose α- and ε-amino groups are coupled to two terminal lysine residues. In fluorescence spectroscopic studies designed to examine relative affinities of binding to the toxin, neutralization of surface charge and fluidization of the acyl domains, the peptide was active, closely resembling the effects of polymyxin B and its nonapeptide derivative; however, the synthetic peptide does not induce phase transitions in LPS aggregates as do polymyxin B and polymyxin B nonapeptide. The peptide was also comparable with polymyxin B in its ability to inhibit LPS-mediated IL-1 and IL-6 release from human peripheral blood mononuclear cells. The synthetic compound is devoid of antibacterial activities and did not induce conductance fluxes in LPS-containing asymmetric planar membranes. These results strengthen the premise that basicity and amphiphilicity are necessary and sufficient physical properties that ascribe endotoxin binding and neutralizing activities, and further suggest that antibacterial/membrane perturbant and LPS neutralizing activities are dissociable, which may be of value in designing LPS-sequestering agents of low toxicity.

Abbreviations

BPI, bactericidal/permeability increasing protein; DC, dansylcadaverine; 5-dimethylaminonaphthalene-1-N-(5-aminopentyl) sulfonamide; DPG, dipalmitoylphosphatidyl -glycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; FITC-LPS, fluorescein isothiocyanate-labeled lipopolysaccharide; IL-1, interleukin-1; IL-6, interleukin-6; LBP, lipopolysaccharide binding protein; LD/TOF, laser desorption/time-of-flight; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PMB, polymyxin B; PMN, polymyxin B nonapeptide; Rh-PE, rhodamine-labeled phosphatidylethanolamine; TNS, 2-(*p*-toluidinyl)naphthalene-6-sulfonic acid, sodium salt.

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INTRODUCTION

The treatment of endotoxic shock, a relatively common and often fatal sequel of systemic Gram-negative bacterial infections, remains non-specific and supportive since specific therapeutic modalities are as yet unavailable.[1–3] Much progress has been achieved during the past two decades in understanding the pathogenesis of endotoxic shock and the convergence of basic research from several seemingly disparate disciplines has spawned diverse experimental approaches aimed at intervening at almost every step, from the binding of LPS to cellular targets to downstream events mediating tissue damage. These efforts, to mention a few, include the use of antibodies,[4] or non-antibody macromolecules that bind LPS,[5–8] nontoxic analogs of lipid A which may act as LPS antagonists,[9–11] agents that block or modulate proximal cellular signaling mechanisms preceding the production of cytokines,[12] and antagonism of cytokine actions.[2,13]

Our laboratories have, in the past few years, sought to explore the possibility of sequestering LPS non-immunologically, using small molecules. This paper, dealing with the endotoxin binding and neutralizing activities of a synthetic peptide is an extension of earlier work on peptides[14–17] and non-peptidic drugs.[18–20] The objectives of the work embodied in this paper are three-fold. Firstly, we had, in the course of examining several basic amphiphilic peptides, proposed[16] that basicity and amphiphilicity may be necessary and sufficient requisites to confer anti-endotoxic properties, and that secondary structural or conformational features in such peptides may not be critical determinants of endotoxin binding or neutralization. Synthetic peptides, designed *ab initio*, provide a direct means of examining this premise. Secondly, the peptides that we had studied[16] are all antimicrobial agents, and it was desirable to examine whether antimicrobial and endotoxin binding and neutralizing activities are dissociable and to rule out the possibility that the anti-endotoxic activities in such peptides were not simply a consequence of their action on Gram-negative bacterial outer membranes. Finally, it has become clear that binding affinity, *per se*, is an unsatisfactory predictor of endotoxin-neutralizing activity, since ligands, while binding endotoxin with comparable affinities may either opsonize or sequester the toxin, human serum albumin[21]/polymyxin B[15,22] and LBP[23–25]/BPI[6,26–28] being examples. It would, therefore, be desirable to identify if other physical parameters of interaction may be adequate predictors of the outcome of ligand binding on the biological activity of LPS, since they may be of potential value in screening compounds for anti-endotoxic activity. We have characterized relative binding affinities, neutralization of the surface charge of LPS, phase state of LPS and of the fluidity of its apolar domain using fluorescence spectroscopic methods, and have attempted to correlate these findings with the biological antagonistic activities in cytokine induction assays. Antimicrobial activity, a consequence of membrane-perturbant properties often found in basic amphiphilic peptides were evaluated in standard antibacterial assays as well as by examining the effects of the synthetic peptide on the conductance of artificial asymmetric planar bilayers containing LPS on one leaflet of the membrane.

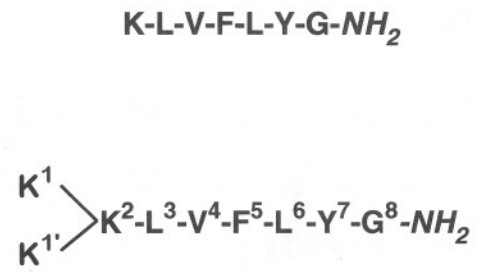

Fig. 1 The peptide sequences of G-2 (bottom), and of its precursor, G-1 (top). Standard one-letter amino acid codes are used. Residues for G-2 are numbered from the N-terminal. The carboxy-termini of the peptides are amidated.

Two cationic amphiphilic peptides were synthesized, designated G-1 and G-2 (Fig. 1). Both peptides possess a common stretch of 6 hydrophobic amino acids. G-1, the precursor compound of G-2, bears a single lysine at the N-terminus, therefore, having two charges at physiologic pH (the α and ε amino groups being protonated) whereas G-2 has two terminal lysines (four charges) branching off from the preceding lysine. Such repetitively branched molecules have been reported in the literature.[29–31] We report, in this paper, the characterization of the interaction of G-2 with LPS and lipid A and of the effects of peptide binding on the cytokine-inducing properties of LPS.

MATERIALS AND METHODS

Reagents

LPS and heptacyl 1,4′-bisphosphoryl lipid A from *Salmonella minnesota* Re595 were in-house preparations and were used as the uniform triethylamine salt forms. Aqueous stock suspensions of lipid A and ReLPS (1 mM) were obtained by sonication for 20 min in a Branson bath-type sonicator at 45°C. Polymyxin B sulfate (PMB), dansylcadaverine (DC), 1,6-diphenyl-1,3,5-hexatriene (DPH), 2-(*p*-toluidinyl)-naphthalene-6-sulfonic acid (TNS), phosphatidylethanolamine (PE) from bovine brain (type 1), phosphatidylglycerol (PG) from egg yolk lecithin

(sodium salt) diphosphatidylglycerol (DPG) from bovine heart (sodium salt), fluorescein isothiocyanate-labeled LPS (FITC-LPS) from *Escherichia coli* serotype 0111:B4, and RPMI-1640 cell culture medium were purchased from Sigma (Deisenhofen, Germany). Phosphatidylethanolamine with the headgroup labeled with rhodamine [N-Lissamine™ rhodamine B sulfonyl)-1,2-dihexadecanoyl -*sn*-glycero-3-phosphoethanolamine, triethylammonium salt] (Rh-PE) was from Molecular Probes (Eugene, OR, USA) and polymyxin B nonapeptide hydrochloride (PMN) was from Boeringher Mannheim (Mannheim, Germany). All Fmoc (9-fluorenylmethyloxycarbonyl)-protected-L-amino acids were procured from Bachem Bioscience (King of Prussia, PA, USA). All other reagents used were of the highest commercial grade available.

Synthesis and characterization of peptides

The peptides were synthesized on an LKB Biolynx semi-automated solid-phase synthesizer using conventional Fmoc chemistry. The sequence of the peptides are shown in Figure 1. In G-2, the branching at the penultimate lysine residue was achieved using α,ε-di-Fmoc-L-lysine; one-step deprotection using 20% piperidine in dimethylformamide yielded two reactive amino groups at the penultimate lysinyl residue for the final coupling step. Cleavage of the peptides from the resin (Novasyn PR-500) was achieved using 10% trifluoroacetic acid in dichloromethane as recommended by the manufacturer (Novabiochem). The use of this resin afforded the synthesis of peptides whose carboxyl termini are amidated, thus eliminating the negative charge and the resultant zwitterionic nature of the peptides. Reverse-phase HPLC on a C_{18} column using a methanol/water/+0.1% trifluoroacetic acid gradient indicated a purity of at least 95%. Laser-desorption/time-of-flight mass spectroscopy (LD/TOF-MS) in the positive ion mode yielded the expected masses of 861 and 971 for the $[M+H_2^{2+}+Na]$ and $[M+H_2^{2+}+Cs]$ adductions, respectively, for G-1 and 1121 and 1231 for the $[M+H_4^{4+}+Na]$ and $[M+H_4^{4+}+Cs]$ adductions, respectively, for G-2. These two peptides were found to be homogenous since extraneous molecular ions were absent in LD/TOF spectra. G-1 and G-2 also yielded the expected molecular-ion peaks at 838 and 1096 mass units, respectively, in electrospray mass spectra.

The first generation precursor peptide (G-1) bearing a single N-terminal lysine residue was poorly soluble in water which is to be expected in view of its dominant hydrophobic character (see Fig. 1) and its pronounced tendency to aggregate under aqueous conditions hindered the preparation of well-dispersed solutions, precluding precise estimations of concentrations by absortion spectrophotometry. For these reasons, we excluded G-1 from our experiments. Concentrations of G-2 in water was determined by absorptimetry at 275 nm using a molar extinction coefficient of 1340 for tyrosine.

Fluorescence experiments

All experiments were carried out in 2 mM Tris-HCl, pH 7.2 at 37°C using a Spex F1 T11 fluorescence spectrophotometer in the photon-counting mode. All spectra were corrected for lamp and instrument characteristics in the ratio-mode. Effective bandpasses were 5 nm for both excitation and emission monochromators in all experiments. Small aliquots of concentrated stock solutions were added in order to minimize dilution effects during titration, and an interval of at least 5 min was allowed to elapse in all titration experiments after each addition of test substances to ensure adequate equilibration. In all experiments, PMB and PMN were also tested as reference compounds.

The use of dansylcadaverine as a fluorescent displacement probe to obtain relative affinities of LPS-binding substances has been described earlier.[15] Briefly, the addition of ReLPS (final concentration: 25 μM) to a solution of DC (50 μM) results in blue-shift (535 nm to 515 nm) and enhancement in the emission spectrum of DC as a consequence of the binding of the probe to the lipid. Sequential addition of a competing ligand displaces bound DC, leading to quenching of fluorescence. The displacement curves were analyzed as a function of added ligand concentration using a four-parameter logistic equation as implemented in the ALLFIT program.[32]

TNS is an anionic fluorescent probe which incorporates into lipid assemblies hydrophobically. The adsorption appears to be governed by electrostatic effects, being an inverse function of the surface charge (Ψ_0) on the lipid matrix. Specifically, the fluorescence intensity is proportional to the concentration of the probe at the lipid surface which, in turn, is inversely proportional to a Boltzmann factor of Ψ_0 and can formally be expressed as: $f = \gamma[F]\exp[-ze\,\Psi_0/(kT)]$ where f is the fluorescence intensity, γ is a proportionality constant, z is the valence of the probe, k is the Boltzmann constant, and T is the absolute temperature.[33] Importantly, the enthalpy of binding of TNS to lipid aggregates is relatively insensitive to the ionic composition of the bulk solvent and to the geometry of the lipid superstructures.[34] These features, in conjunction with relatively large blue-shifts and quantum yield increases upon binding to membranes, render TNS a suitable probe to monitor changes in electrostatic potentials and has been employed to probe the binding of monovalent cations[33] and basic peptides[34,35] to negatively charged membranes. However, a significant disadvantage with this method is that TNS binds much more avidly to membranes in the fluid phase than in the gel phase.[36] This impedes a quantitative assessment of the change of Ψ_0 in

systems in which there is a concomitant phase transition such as encountered in experiments reported in this paper (see below). We, therefore, did not perform numerical computations of Ψ_0, restricting ourselves to qualitative comparisons of G-2 with the reference compounds, PMB and its nonapeptide derivative. In these experiments, a methanolic solution of TNS was added to 1.0 ml buffer in a quartz cuvette (final concentration: 75 μM). ReLPS was then added to a final concentration of 30 μM. An excess of TNS was used in order to ensure that the concentration of the probe was not limiting. Excitation was at 320 nm. The emission maximum of free aqueous TNS in the absence of either LPS or peptide was at 480 nm, and shifted to 440 nm with addition of ReLPS, concomitant with an approximately two-fold intensity enhancement, which is consistent with partial incorporation of TNS in the lipid. Upon titration with peptide, a further blue-shift of about 15 nm with typically 10–15-fold intensity enhancement occurred, indicative of increased TNS binding. The results are reported as $F - F_0/F_0 \times 100$, where F and F_0 are the intensities at 425 nm at a given peptide concentration, and in the absence of peptide, respectively.

The effect of G-2 on the fluidity of lipid A suspensions was examined using the steady-state emission polarization of DPH.[18] A mixture of lipid A or ReLPS and DPH (100:1 molar ratio) in chloroform was evaporated in a stream of dry N_2 and reconstituted in buffer by sonication. A final lipid A or ReLPS concentration of 25 μM in 1.0 ml buffer was used. Signals at 430 nm (excitation: 360 nm) of vertically and horizontally polarized emission were simultaneously acquired for 60 s for each titration step at a data sampling interval of 0.1 s using a dual orthogonally-oriented photomultiplier. Polarization was calculated as $(g.I\|\text{-}L\perp)/(g.\ I\| + L\perp)$ where $L\perp$ and $I\|$ are emission intensities with the excitation and emission polarizers aligned parallel and perpendicular respectively, and g is the instrument correction factor, given by $L\perp/I\|$. Polarization values were averaged over 60 s and the coefficient of variation was typically < 0.5 %.

The possibility of peptide-induced bilayer-to-nonlamellar phase transitions was evaluated using ReLPS doped with Rh-PE. Neutral phospholipids with the headgroup labeled with suitable fluorophores incorporated in membrane suspensions have been shown to be sufficiently sensitive to such phase transitions[37,38] and are usually manifested as discontinuities in temperature or binding isotherms.[37] When changes in the supramolecular state are accompanied by $\beta \rightarrow \alpha$ transitions of the hydrocarbon region as is the case for LPS and lipid A,[39–41] the two processes can be resolved by following the intensity changes of the probe: whereas liquid crystalline-to-fluid transitions are characterized by decrease in intensity as a result of greater lateral mobility of the probe and consequent self-quenching phenomena, lamellar-to-nonlamellar transitions are distinguished by intensity enhancements, presumably because of changes in the dielectric environment in the immediate vicinity of the fluorophore. Mixtures of ReLPS with Rh-PE (0.1 mole %) in $CHCl_3$ were evaporated and reconstituted in buffer as described above. Emission spectra from 560 nm to 640 nm were obtained after each addition of peptide with excitation at 530 nm. Changes in intensity were analyzed as a function of added peptide concentration.

Conductance measurements in asymmetric planar bilayer systems

Several basic amphiphilic peptides exhibit antimicrobial properties by virtue of their ability to interact with, and perturb bacterial membranes[6,16,42–48] and it was therefore of interest, as an adjunct to conventional antibacterial screening techniques, to examine the effect of the synthetic peptide on reconstituted bacterial membrane systems. Conductance fluxes across asymmetric planar membranes were measured as described earlier.[49–52] Briefly, asymmetric membranes were prepared by sequentially apposing two differently constituted lipid monolayers (*S. minnesota* ReLPS on one monolayer, and a mixture of neutral and anionic phospholipids on the other) over an aperture of 150 μm diameter. The phospholipid mixture comprised of PE, PG and DPG in a molar ratio of 81:17:2. The subphase bathing the membrane in these experiments contained, in addition to 50 mM KCl, 5 mM $MgCl_2$ and 5 mM HEPES buffer, pH 7.4 as reported in earlier studies, 4% dimethylsulfoxide. The presence of low concentrations of DMSO conferred greater resistance against irreversible membrane breakdown and allowed much greater time periods of data acquisition following peptide addition. This effect, observed fortuitously, and perhaps related to nonspecific membrane stabilizing effects[53–55] did not confound conductance measurements, or affect membrane capacitance (140–160 pF), and the results obtained with PMB in the presence of DMSO in the present experiments are consistent with the extensive characterization performed in the past.[49–52] The membranes were voltage-clamped via Ag/AgCl electrodes which were connected to the headstage amplifier (1 GΩ impedance) of a BLM 120 bilayer membrane amplifier (BioLogic, Claix, France). Voltages were applied to one electrode, the other being grounded. Voltage pulses were maintained for at least 60 s for each increment, thereby ensuring optimal steady-state measurements. Current fluxes and voltages were stored on a DAT-tape recorder interfaced to the amplifier through a 4-pole low-pass Bessel filter. Data were downloaded after completion of the experiment on an AT-compatible computer and were analyzed at a sampling frequency of 10 Hz. Current flow was designated positive

when cation flux was directed toward the grounded compartment. All experiments were carried out at 37°C.

Effect of the peptide on cytokine-inducing activity of LPS

The effect of complexation of the peptide with LPS on IL-1 and IL-6 inducing potential of the latter were examined in human peripheral blood mononuclear cells. PBMC, obtained from healthy donors (10^6/ml in RPMI 1640 containing antibiotics and 10% autologous human serum) were incubated with 10 ng/ml of LPS in the absence or presence of varying amounts of peptide for 8 h in 5% CO_2 at 37°C in U-form microtiter plates. Polymyxin B was used as a positive control for inhibition of cytokine production. Supernatants were harvested and analyzed for cytokine activity in bioassays either immediately or after storage at −20°C. IL-1 activity was quantified using dermal fibroblasts isolated from human foreskin.[56] IL-6 bioactivity was determined using an IL-6-dependent B9.9-3A4 cell proliferation. Cytokine levels are reported as units of activity/ml and are referenced to human recombinant cytokines of known activity.

Screening for microbicidal activity

Microbicidal activities were assayed using conventional Kirby-Bauer disk-diffusion methods against *E. coli*, strain ATCC 11775. Aliquots (5 µl) of the test substance serially 5-fold diluted from 300 µg/ml to 0.3 µg/ml were tested in quadruplicate. Polymyxin B was used as the reference antibiotic.

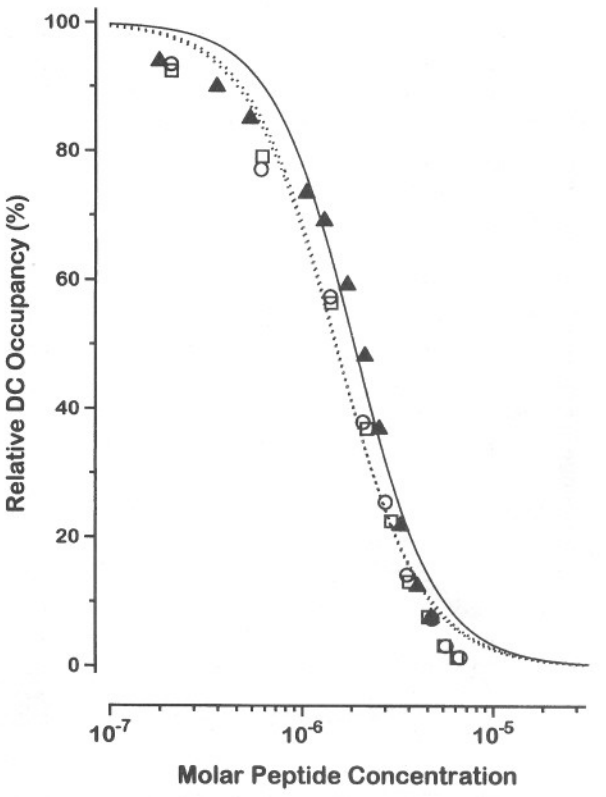

Fig. 2 Displacement of dansylcadaverine from *S. minnesota* Re 595 LPS. G-2: filled circles; solid line. Control substances (interrupted lines and open symbols) PMB: open squares; PMN: open circles. Successive aliquots of peptides were added to the mixture of ReLPS (25 µM) and fluorescent probe (50 µM) in 2 mM Tris-HCl, pH 7.2, at 37°C. The X-axis represents the final molar concentrations of the peptides. Values of relative probe occupancy denoted on the Y-axis were calculated as described.[15] Displacement curves were fit by ALLFIT (*see* Materials and Methods).

RESULTS

Fluorescence experiments

In the dansylcadaverine displacement assay, used as a preliminary biophysical screen to estimate the relative affinity of binding of the peptide, the apparent dissociation constants for PMB, PMN and G-2 are very similar (Fig. 2), the respective ID_{50} values computed by the ALLFIT method being 1.51, 1.49, and 1.67 µM.

In experiments with TNS designed to observe changes in surface potential of LPS aggregates upon peptide addition, TNS incorporation was analyzed as a function of the peptide:LPS molar ratios (Fig. 3). The addition of either PMB or PMN led to an increase in the incorporation of TNS, indicative of peptide binding and consequent neutralization of the negative charge on the lipid. The TNS intensity enhancement is strikingly greater for PMB than for PMN. As mentioned earlier, the phase state of the lipid is also a determinant of TNS binding and we attribute this difference to the greater fluidization induced by PMB (see below). The intensity profile appears to follow a Langmuir-type isotherm with an apparent saturation in TNS fluorescence at a peptide:LPS molar ratio of about 1:1 for both PMN and PMB. Since the concentration of TNS was not limiting in these experiments, the inflections denote the stoichiometry of the complex. The reason for the second inflection observed for polymyxin B at 1.5:1 molar ratio is unclear and presumably involves reorientation of the geometry of the complex at the higher peptide concentration. Direct zeta potential measurements by laser Doppler anemometry also yielded a stoichiometry of about 1:1 for the PMB-ReLPS complex (data not shown). G-2 also attenuates the surface charge on the LPS superstructures, and an apparent stoichiometry of 1:1 is observed for the interaction (Fig. 3).

We analyzed the effect of peptide binding on the fluidity of the apolar domain of lipid A and ReLPS and the

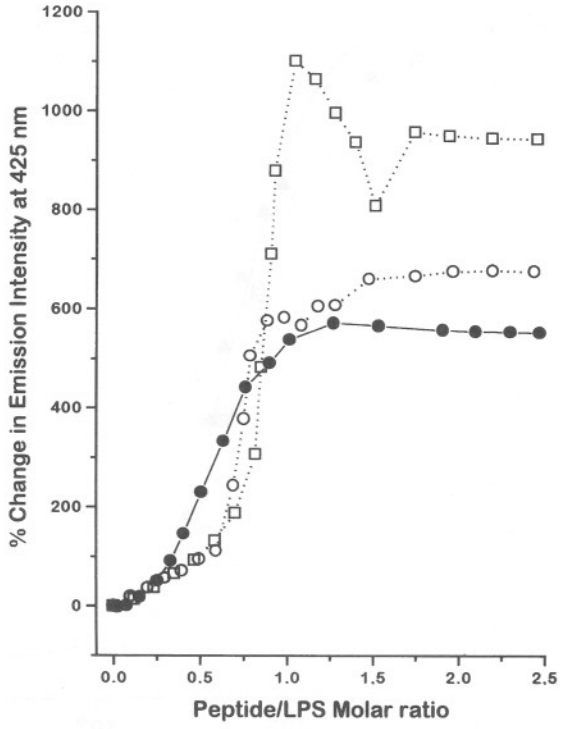

Fig. 3 Enhancement of TNS emission intensity at 425 nm upon addition of peptides. G-2: filled circles; solid line. Control substances (interrupted lines and open symbols) PMB: open squares; PMN: open circles. Aliquots of peptides were incrementally added to a mixture of ReLPS (30 μM) and TNS (75 μM) in 2 mM Tris-HCl, pH 7.2 at 37°C. Excitation was at 320 nm.

results obtained for lipid A with steady-state fluorescence polarization spectroscopy using DPH are presented in Figure 4. Both PMB and PMN increase the rotational mobility of DPH incorporated in lipid A suspensions resulting in decreased polarization values, PMB being more effective than PMN which is likely a consequence of the penetration of the hydrophobic tail of PMB. Similar differences were also obtained in experiments employing the diffusion-controlled excimerization of pyrene incorporated into lipid A[20] as well as by direct FT-IR measurements of the CH_2–vibrational modes of the hydrocarbon region (data not shown). The primary inflection of the polarization profiles for both PMB and PMN appear to correspond to a peptide:lipid A molar ratio of 1:2, which is identical to independent determinations using TNS (data not shown). Although G-2 is effective in reducing the order of the acyl chains, the interaction appears to be distinctly biphasic with fluidization at molar ratios of less than 1, followed by a reversal of the change. Two main inflections are noted at 1:2 and 1:1 molar ratio of G-2:lipid A, possibly signifying structural polymorphisms of the lipid-peptide assemblies at different peptide concentrations. The reversal of fluidization at higher G-2 concentrations is particularly intriguing. Three possibilities were considered in an attempt to rationalize this observation: (i) a lamellar-to-non-lamellar phase transition; or (ii) a lateral phase separation of the lipid with the formation of microdomains with highly ordered acyl regions; and (iii) a 'solubilization' of the lipid A and consequent loss of the lipid from the aggregate. While it is not possible to unambiguously distinguish between the these possibilities on the basis of the available data, the first process appears unlikely since we could not detect any such transitions in Rh-PE doped LPS preparations (see below). A solubilization effect may be tenable since light scattering intensities decreased upon adding the peptide and were considerably lower at the end of the titration than at the beginning in contrast to either PMB or PMN, with which macroscopic precipitates were observed at sufficiently high peptide concentrations. Further experiments are necessary to clarify the physical basis of this observation.

In experiments performed with the neutral rhodamine-labeled PE incorporated into ReLPS at low probe concentrations, PMB and PMN induce a biphasic change in intensity (Fig. 5). The initial phase of intensity enhancement is probably a consequence of a lamellar-to-non-

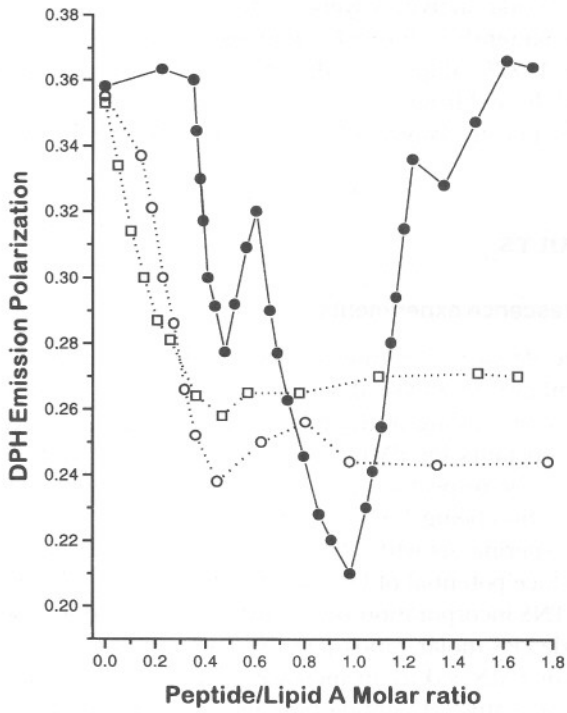

Fig. 4 Effect of peptides on the steady-state emission polarization of DPH incorporated into lipid A. G-2: filled circles; solid line. Controls (interrupted lines and open symbols) PMB: open squares; PMN: open circles. [Lipid A]: 25 μM; [DPH]: 0.25 μM. Temperature: 37°C. Excitation and emission wavelengths were 360 and 430 nm, respectively.

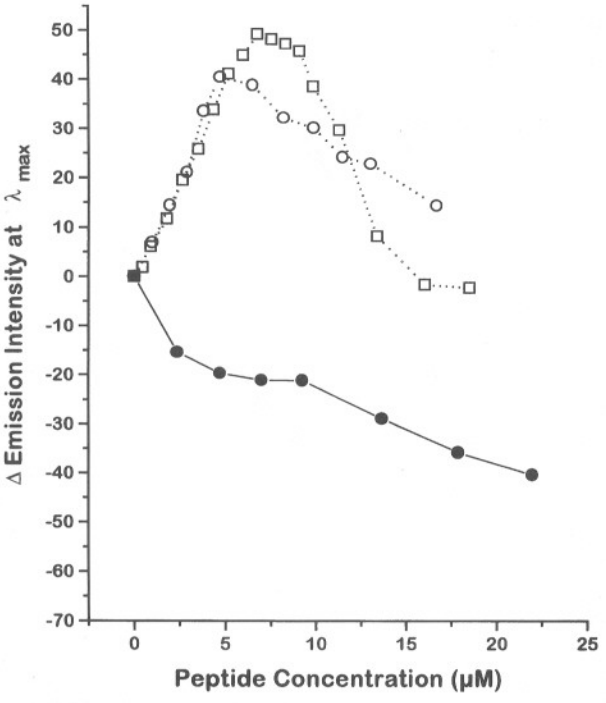

Fig. 5 Effect of peptides on the emission of ReLPS doped with Rh-PE. G-2: filled circles; solid line. Controls (interrupted lines and open symbols) PMB: open squares; PMN: open circles. [Lipid A]: 25 μM. Excitation was at 530 nm. Changes in emission intensity at λ_{max} are plotted against peptide concentration.

lamellar phase transition. At higher concentrations of either PMN or PMB, phase separation of the neutral probe and subsequent self-quenching effects supervene, resulting in intensity attenuation. The synthetic peptide does not appear to induce such phase-transitions since its interaction results only in a concentration-dependent quenching (Fig. 5), indicative, as mentioned earlier, of lateral phase separation of the probe.

Effect on conductance in Re-LPS-containing asymmetric bilayers

The membrane conductance-inducing behavior of the two reference peptides PMB and PMN were first characterized and representative data are shown in Figure 6. Polymyxin B at 5 μM induces large bidirectional voltage-dependent current fluxes. The addition of PMN (20 μM) does not result in current fluxes significantly different from basal fluxes at ± 80 mV applied voltage (Fig. 6). These results are in accordance with earlier observations.[49] Figure 7 depicts the measurements performed with G-2. The synthetic peptide does not show any intrinsic effect on conductance even at 50 μM. When PMB was subsequently added to a final concentration of 10 μM and the voltage pulse program was reapplied, changes in conductance characteristic of PMB action are observed (Fig. 7). The magnitude of the PMB-induced current fluctuations are lower than those recorded in the absence of prior synthetic peptide addition, which is to be expected

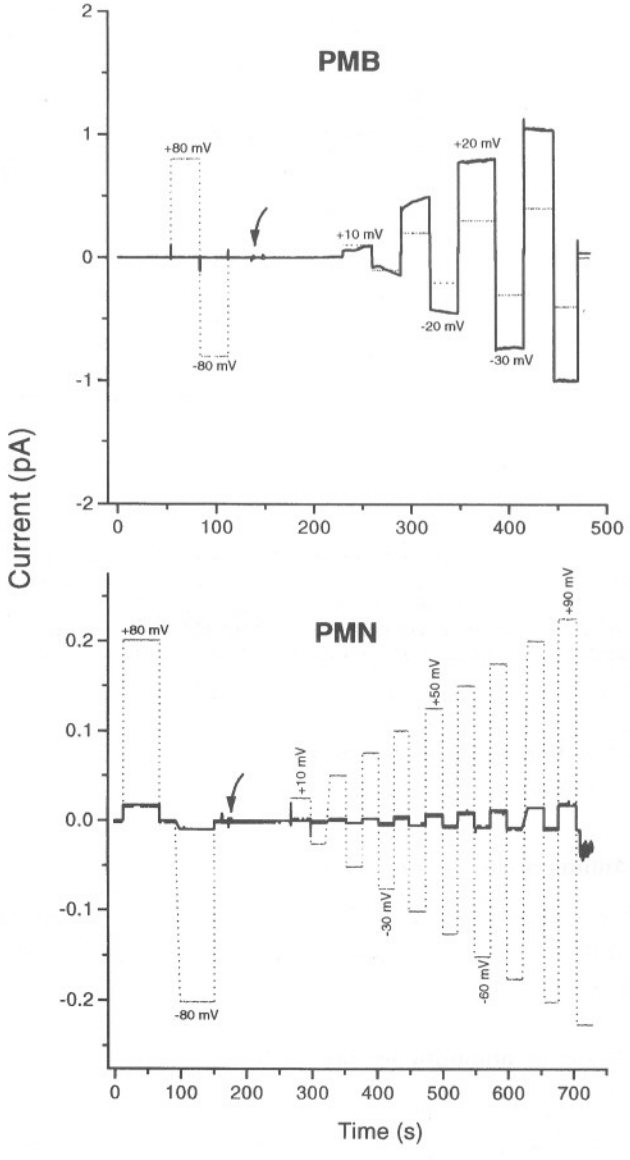

Fig. 6 Effect of polymyxin B (positive control; top panel) and polymyxin B nonapeptide (negative control; bottom panel) on conductance fluxes in ReLPS/phospholipid asymmetric planar membranes. Applied voltage pulses (in mV) are denoted in dotted lines and are scaled arbitrarily. Current fluxes (in pA) drawn in solid lines correspond to the Y-axis scale. A bipolar 80 mV pulse was first applied after membrane constitution to confirm membrane stability. The peptide (PMB: 5 μM, top panel; PMN: 20 μM, bottom panel) was then added, denoted by the curved arrows. After an equilibration period of at least 100 s, bipolar voltage pulses were applied in 10 mV increments. Note the response to PMB even at low applied voltages, and the refractoriness to PMN (Y-axis scale of bottom panel magnified). Experimental conditions are described in detail in Materials and Methods. Data from a single representative experiment are depicted.

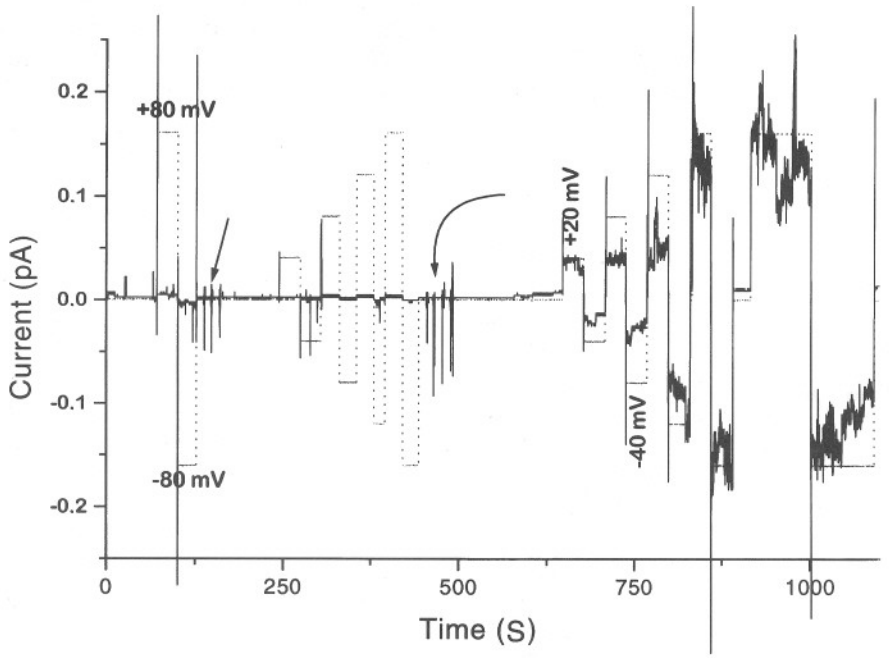

Fig. 7 Effect of G-2 on conductance across ReLPS/phospholipid asymmetric planar membranes. Applied voltage pulses (in mV) are denoted in dotted lines and are scaled arbitrarily. Current fluxes (in pA) drawn in solid lines correspond to the Y-axis scale. The straight arrows following the initial 80 mV verification pulse denotes the addition of G-2 (final concentration: 50 μM). After an equilibration period of ~100 s, bipolar voltage pulses of ± 20, 40, 60 and 80 mV were applied. The spikes in the current trace are artifactual switching transients. To confirm the integrity and responsiveness of the membrane preparations following treatment with G-2, polymyxin B (10 μM) was subsequently added (curved arrows) and the voltage-pulse program reapplied.

since both substances would compete for binding at the membrane.

Antimicrobial activity

G-2 was not microbicidal to *E. coli* at the highest concentration tested (300 μg/ml). Significant inhibition zones were observed at 0.3 μg/ml for PMB.

Cytokine inhibitory activity

In the IL-1 and IL-6 release assays, G-2 was as potent as PMB (Fig. 8). In control experiments, none of the peptides induced cytokine production in the absence of LPS at the highest concentration tested (50 μM).

DISCUSSION

Our previous observations that several substances of diverse classes and disparate structures, being similar only in possessing basic and hydrophobic properties, bind and attenuate LPS toxicity suggested that compounds structurally unrelated to polymyxin B may behave as functional analogs of PMB, a premise, if valid, may provide potentially useful leads in the design of nontoxic and therapeutically useful polymyxin B-like substances which may be employed to sequester endotoxin. The synthetic oligopeptide, G-2, represents a step toward the rational design of such analogs. The branched, and splayed cationic terminus of G-2 was designed with a view to providing a spatial distribution of positive charge similar to that of small cyclic peptides such as PMB, and, in being essentially linear, enabled a straightforward synthetic approach which, in the case of cyclic peptides is usually a nontrivial problem. A recent study on the binding of PMB to lipid A and LPS using isothermal titration calorimetry[22] suggested a dominant role for hydrophobic interactions in the formation of the complex. The hydrophobic nature of PMB is a consequence of both an asymmetric arrangement of polar and apolar amino acids as well as the presence of a terminal methyloctanoyl acyl moiety. In order to functionally mimic the hydrophobic character of PMB (and thereby also enhance amphiphilicity) all residues in G-2 other than the C-terminus (glycine) and the N-terminal lysines are apolar. Most linear membrane-active peptides are about 18 residues or longer, and in order to minimize possible membrane-perturbant activities of the peptide, the length of its C-terminal hydrophobic stretch was limited to 6 residues.

It is clear from the data presented above that G-2 resembles closely the effects of PMB and PMN with regard to its interaction with lipid A or LPS and, importantly, attenuates LPS activity in cytokine release assays with a potency comparable to the reference compounds. The binding affinities of G-2 and the reference compounds are virtually identical, and all three compounds diminish, upon binding, the surface potential on ReLPS aggregates, and the state of order of the hydrocarbon regions of lipid A. In experiments using TNS, PMB and PMN bind to ReLPS with a 1:1 stoichiometry, consistent with the charge ratios (four or five negative charges in ReLPS and five positive charges in PMB or PMN), and is in agreement with values determined independently using Langmuir monolayer film balance and zeta potential measurements (unpublished data). G-2 behaves in a similar manner, the observed stoichiometry being also about 1:1 as expected in view of its 4 charges. In the DPH experiments performed with lipid A, the addition of either PMB or PMN resulted in the expected increase in fluidity, consistent with earlier reports in the literature[57–61] with the expected peptide:lipid A molar ratios of 1:2. The inflections observed in the polarization measurements corresponding to G-2/lipid A molar ratios at 0.5:1, 1:1, and 1.5:1 suggest a certain 'plasticity' in its recognition of lipid A, which is presumably a consequence of the intrinsic conformational flexibility of the peptide and a clear resolution of the structural basis of the variable stoichiometry must await further work using conformationally constrained peptides. Collectively, these data rule out a nonspecific 'adsorption' of the oligopeptide as has been observed with polycations[62] and suggest that the antagonism of the biological activity of LPS, examined in the present study using cytokine release, is a consequence of the formation of specific, stoichiometric complexes of G-2 with lipid A and ReLPS.

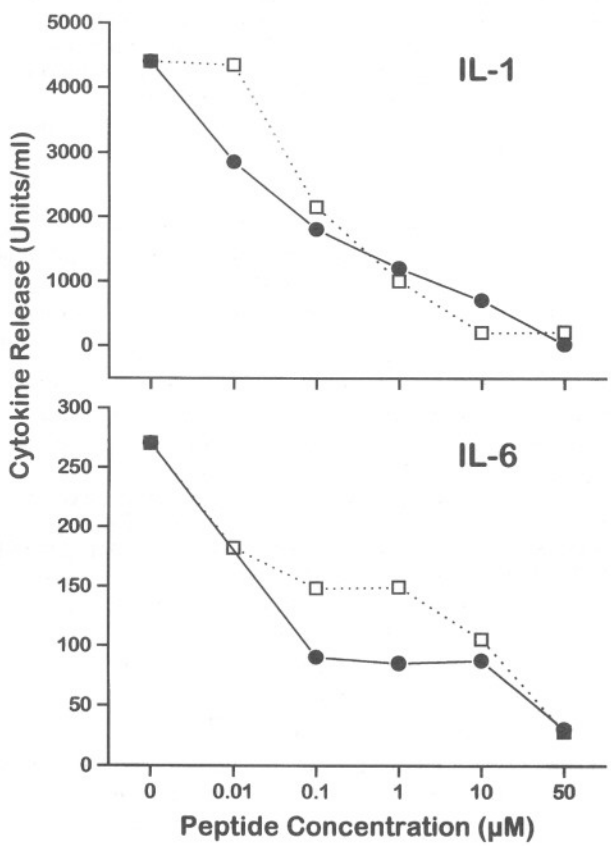

Fig. 8 Dose-dependent inhibition of IL-1 (top panel) and IL-6 (bottom panel) from human PBMC stimulated with 10 ng/ml LPS in the presence of serum. G-2: filled circles; PMB (control): open squares. Means of quadruplicate determinations of a single representative experiment are shown.

Unlike PMB and PMN, however, G-2 is incapable of inducing polymorphic phase transitions in lipid A aggregates when examined using rhodamine-phosphatidylethanolamine as a probe. G-2 is also devoid of microbicidal activity against Gram-negative bacteria and does not elicit electrically measurable permeability changes in reconstituted Re-LPS-containing planar bilayers. The structural requirements for ligands to induce lamellar-to-nonlamellar phase transitions in LPS are as yet unknown and it would be instructive to examine if other compounds known to affect the integrity of bacterial outer membranes also exhibit such transition-inducing effects as have been observed in these experiments.

Finally, it would be appropriate to relate the present results to those reported in the literature on other LPS-binding peptides. In studies on synthetic peptide analogs of polymyxin B[63] and tachyplesin I[64], several compounds, linear as well as disulfide-cyclized, showed significant inhibition of LPS activity in the *Limulus* gelation and murine in vivo TNF and IL-6 induction assays, the only peptides failing to inhibit LPS-induced biological responses being either very short, or feebly basic. These results are consonant with the proposition that strict structural or stereochemical requisites are probably not critical in LPS-binding ligands. The considerable affinity and LPS-neutralizing activity together with an anticipated low degree of toxicity renders G-2 an attractive lead. Further investigations on the dependence of activity on the overall length, charge density, and the nature of the C-terminal stretch (hydrophobic vs amphiphilic) are in progress. Such peptides with a higher degree of branching ('dendrimers'[29–31]) may be of particular interest.

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