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Biochimica et Biophysica Acta 1212 (1994) 167–175

BBA Biochimica et Biophysica Acta

Interaction of cationic amphiphilic drugs with lipid A: Implications for development of endotoxin antagonists

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(Received 18 August 1993)

Abstract

This report presents evidence for the interactions of several classes of cationic amphiphilic drugs including the phenothiazines, aminoquinolines, biguanides, and aromatic diamidines, with lipid A, the endotoxic principle of lipopolysaccharides. The interactions of the drugs were quantitatively assessed by fluorescence methods. The affinities of the drugs for lipid A parallel their endotoxin-antagonistic effects in the *Limulus* gelation assay. Dicationic compounds bind lipid A with greater affinity; the affinity of such molecules increases exponentially as a function of the distance between the basic moieties. The bis-amidine drug – pentamidine – examined in greater detail, binds lipid A with high affinity (apparent K_d: 0.12 μM), and LPS, probably due to simultaneous interactions of the terminal amidine groups with the anionic phosphates on lipid A. The sequestration of endotoxin by pentamidine reduces its propensity to bind to cells, and the complex exhibits attenuated toxicity in biological assays. These results have implications in the development of therapeutic strategies against endotoxin-related disease states.

Key words: Lipopolysaccharide; Lipid A; Fluorescence; NMR; Phenothiazine; Aminoquinoline; Biguanide; Pentamidine; Endotoxin-antagonism

1. Introduction

Many of the pathological consequences of septic shock are attributable to endotoxins or lipopolysaccharides (LPS), structural components of the outer membrane of gram-negative organisms [1]. The mechanisms leading to endotoxic shock are mediated by a cascade of host responses to LPS, important among which are the elaboration of proinflammatory cytokines including tumor necrosis factor and various interleukins by monocytes [2], ultimately manifesting in the shock syndrome. Mortality due to septic shock remains virtually unchanged over the past decade at around 70% [3], reflecting, in part, the absence of specific anti-endotoxic therapy, and has provided a strong impetus for the development of several experimental approaches targeting specific processes underlying this common clinical entity [4]. The amphiphilic lipid portion of LPS, termed 'lipid A' elicits most of the toxic effects of LPS and therefore represents the toxic center of the endotoxins [5]. One possible therapeutic approach aimed at abrogation of endotoxicity can therefore be directed toward neutralization of lipid A activity. Polymyxin B, a cationic cyclic decapeptide antibiotic binds lipid A [6], and the resultant complex is virtually inactive (see Ref. 7 and references therein). The toxicity of polymyxin, however, precludes its use in the clinical situation. In an attempt to understand the structural

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Abbreviations: DC, dansylcadaverine (5-dimethylaminonaphthalene-1-(*N*-(5-aminopentyl)sulfonamide; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoylphosphatidylcholine; LPS, lipopolysaccharide; FITC-LPS, fluorescein isothiocyanate-labelled lipopolysaccharide; LAL, *Limulus* amebocyte lysate; NOESY, nuclear Overhauser enhancement spectroscopy; PBS, phosphate-buffered saline.

SSDI 0005-2760(93)E0262-I

features of polymyxin B which confer endotoxin-antagonist properties, we studied a series of basic amphiphilic peptides of diverse sequences including melittin [8], gramicidin S, tyrocidin and efrapeptin [9]. From these studies we concluded that, in general, cationic amphiphiles may possess endotoxin-antagonistic properties. We now report that various classes of cationic amphiphilic drugs already in therapeutic use bind lipid A and LPS, resulting in attenuated endotoxicity.

2. Materials and methods

2.1. Reagents

Lipid A (diphosphoryl, hexacyl type) from *E. coli* K12 D31m4 was purchased from List Biologicals (Campbell, CA). Mono- and di-phosphoryl lipid A from *Salmonella minnesota* Re595 was obtained from Ribi Immunochem Research (Hamilton, MT). LPS and fluorescein isothiocyanate-labelled LPS (FITC-LPS) from *E. coli* 026:B6, chloroquine diphosphate, primaquine diphosphate, chlorpromazine HCl, promethazine HCl, trifluoperazine 2HCl metformin HCl, phenformin HCl, chlorhexidine 2HCl, pentamidine isethionate, polymyxin B sulfate, dimyristoylphosphatidylcholine (DMPC), 1,6-diphenyl-1,3,5-hexatriene (DPH), pyrene, 1,3-diaminopropane, putrescine, cadaverine, 1,6-diaminohexane, 1,8-diaminooctane, spermidine, spermine, *Limulus* amebocyte lysate (LAL), endotoxin-free water, endotoxin reference (*Shigella flexneri* LPS), RPMI-1640 culture medium and fetal bovine serum were from Sigma Chemical (St. Louis, MO). Dansylcadaverine (5-Dimethylaminonaphthalene-1-(*N*-(5-aminopentyl)) sulfonamide, DC) was synthesized as reported earlier [10]. Concentrations of DC in water were determined by spectrophotometry at 330 nm using a molar extinction coefficient of 3300 for the dansyl moiety. Lipid A was solubilized in water containing 0.5% triethylamine by vigorous vortexing and heating the suspension above the phase transition temperature of lipid A (60°C) for 10 min.

2.2. Fluorescence experiments

Fluorescence methods were employed as an initial biophysical screen to assess the binding of the drugs to lipid A and to compare their relative affinities. Experiments were performed in 10 mM phosphate-buffered saline, pH 7.4 (PBS) using a Shimadzu RF-5000 spectrofluorimeter at 25°C (below the phase-transition temperature of lipid A). Bandpasses for excitation and emission were 5 nm in all experiments. The use of DC as a fluorescent displacement probe to characterize the binding of basic molecules to lipid A has been described earlier [11]. 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 displace DC, resulting in quenching of fluorescence. Probe displacement is analyzed as a function of displacer concentration from which the apparent dissociation constants of the ligands for lipid A are computed by the method of Horovitz and Levitzki [12]. Comparisons of the DC displacement curves to evaluate relative potencies of the compounds were performed by simultaneous curve-fitting with a four-parameter logistic equation to obtain accurate ED_{50} values using the ALLFIT program [13]. For the DC displacement experiments, the excitation wavelength was 340 nm. Emission spectra were obtained by scanning from 400 nm to 600 nm. The wavelengths of maximal emission of free and lipid-bound DC in buffer were 535 nm and 515 nm respectively. The lipid A concentration was 7 μM. The probe concentration was 45 μM in order to ensure maximal ligand occupancy [11]. None of the compounds evaluated produced inner filter effects at the concentrations used as verified by absorbance measurements at 340 nm. That the quenching of lipid A-bound DC fluorescence upon addition of drug was due to probe displacement and not due to filter effects was also evident from the gradual red-shifting of DC emission with increasing concentrations of displacer, which, at the end of the titration, resulted in emission spectra which resembled that of free DC in buffer. Furthermore, the drugs alone had no effect on DC fluorescence in control experiments.

DC was unsuitable in characterizing the binding of phenothiazines to lipid A because of considerable overlap in the emission spectra of phenothiazines [14] and the probe. The intrinsic fluorescence of the drugs could not be used because of marked photolability. We therefore used DPH, a lipid-soluble, neutral fluorescent probe which partitions into the hydrophobic regions of lipids resulting in fluorescence enhancement. A 1 μM DPH suspension in water was prepared by adding an aliquot of 2 mM DPH stock solution in tetrahydrofuran and stirring it vigorously overnight. An aliquot of lipid A was then added such that its final concentration was 10 μM. Excitation was at 360 nm and emission intensities were recorded at 430 nm. The emission intensities of DPH in water before and after addition of lipid A represented F_o and F_{max}, respectively. Successive aliquots of phenothiazine solutions in water were then added. The phenothiazines quench DPH fluorescence as reported elsewhere [15] and the data were analyzed by conventional Stern-Volmer (SV) plots. Chloroquine could not be characterized by either method because of its intense intrinsic fluorescence which confounded both DC and DPH spectra; the intrinsic fluorescence of the drug could not be employed because of photolability.

Concentrations of the hygroscopic diaminoalkanes and organic polyamines in solutions were estimated fluorimetrically by assaying primary amino groups using fluorescamine [16]. The effect of the drugs on the fluidity of the apolar region of lipid A was examined by fluorescence methods employing the diffusion-controlled excimerization of pyrene [17].

2.3. Biological assays

Endpoint LAL gelation, splenocyte proliferation, and chick embryo lethality assays were performed exactly as described previously [8]. In the LAL assay which was employed as a preliminary in vitro screen for biological antagonism, 10:1 drug/lipid A molar ratios were used since this provided a wider dispersion of liquid-to-gel endpoints, and therefore facilitated comparisons of antagonistic potency. Compounds which displayed significant antagonism were re-examined at equimolar ratios. Solutions of the drugs were prepared in endotoxin-free water and were verified to have no intrinsic LAL-gelation activity in control experiments. The latter two biological assays and the experiments described below were performed using pentamidine.

2.4. NMR spectroscopy

In these experiments, purified LPS, rather than lipid A was used because of aqueous insolubility of the latter in the absence of triethylamine. ^1H nuclear magnetic resonance spectra were recorded with a Bruker AM 400 spectrometer at 400 MHz. Measurements were carried out at 25°C in D_2O (99.96%; Sigma) with tetramethylsilane as external standard. Small aliquots of a concentrated stock suspension of *E. coli* 026:B6 LPS in D_2O were successively added to 5 mM pentamidine isethionate in D_2O. 2D NOESY spectra of a sample containing 5 mM pentamidine and 2 mg LPS were acquired in the phase-sensitive mode at mixing intervals of 100 ms with 450 time increments.

2.5. Flow cytometry

FITC-LPS (25 μg) was incubated with either saline or approx. 10 molar excess of either pentamidine or polymyxin B for 2 h at 37°C in the dark. Spleen cells ($5 \cdot 10^6$/ml) from BALB/c mice were then labelled with FITC-LPS/drug mixtures or untreated FITC-LPS in RPMI at 37°C for 3 h in the dark, washed twice in RPMI and resuspended in 0.5% paraformaldehyde in PBS. Flow cytometry experiments were performed with a FACScan instrument (Becton and Dickinson, Mountain View, CA). Rectilinear forward angle light scatter and side light scatter gates were used to define the cell population. Autofluorescence threshold levels were set on unlabelled cells. 10^4 cells were analyzed in each sample. The binding of FITC-LPS to the cells were analyzed by statistical (Kolmogorov-Smirnov) comparisons of frequency distribution histograms of fluorescence intensity.

2.6. Determination of interatomic distances

The DISCOVER molecular modelling software (version 2.51; Biosym Technologies) running on a Silicon Graphics Iris workstation was used. The interphosphate distance in an *E. coli* (hexacyl) diphosphoryl lipid A model was computed to be 13.2 Å from atomic coordinates provided by Prof. Kastowsky (Institut für Kristallographie, University of Berlin) [18]. The interni-

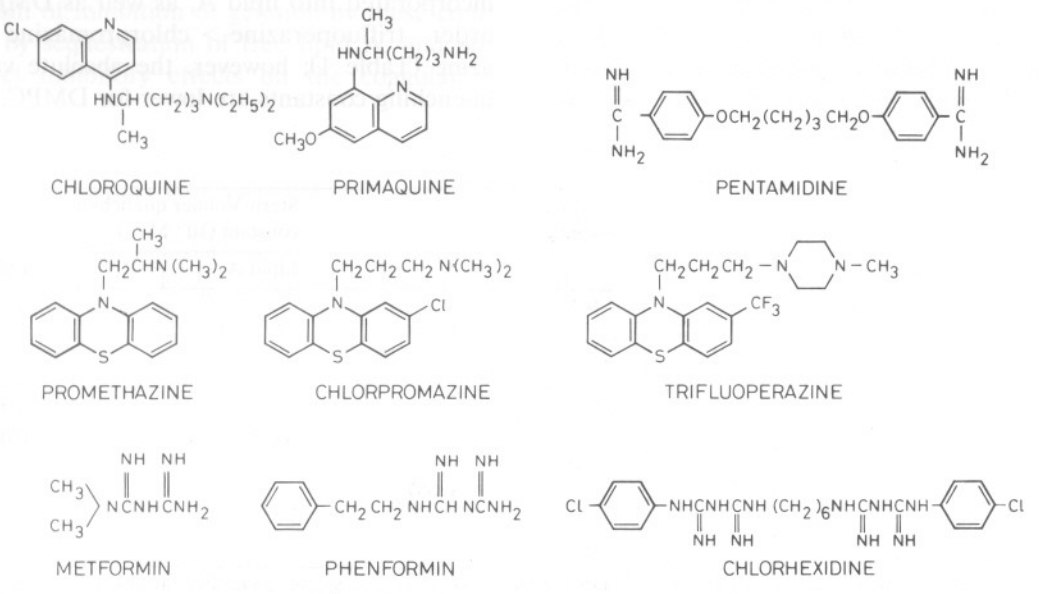

Fig. 1. Chemical structures of cationic drugs.

trogen distances in pentamidine (18 Å) [19] and spermidine [20] were calculated from crystallographic coordinates and those of diaminoalkanes and polyamines were obtained from models constructed using standard bond lengths and angles from the structure fragment libraries in DISCOVER. The internitrogen distance for spermidine obtained by modelling was 11.17 Å, and from crystallographic coordinates [20], 11.15 Å.

3. Results

3.1. Fluorescence experiments

The molecular structures of the drugs are shown in Fig. 1. The dansylcadaverine displacement profiles of polymyxin B, primaquine, metformin, phenformin, chlorhexidine, and pentamidine are shown in Fig. 2. ED_{50} values and empirical dissociation constants derived from the fluorescent probe displacement experiments represented in Table 1 are indicative of high affinity interactions of pentamidine with lipid A. The ED_{50} values for pentamidine obtained in DC displacement assays are very similar for mono- and di-phosphoryl heptacyl (*Salmonella*) lipid A, the ratios being 1:1.14 (data not shown). The affinity of the bis-biguanide, chlorhexidine, is greater than the biguanide compounds metformin and phenformin. Since the affinity of interaction appeared to correlate with the presence of two charged groups separated by a distance, further experiments were carried out with a series of diaminoalkanes and organic polyamines to systematically examine the relationship between internitrogen distance and affinity toward lipid A. The data presented in Fig. 3 indicate that the affinity increases exponentially with the inter-nitrogen distance between 5–16 Å. The affinities of spermidine (11.2 Å) and 1,8-diaminooctane (11.5 Å) are very similar, signifying that the presence of additional non-terminal secondary amino groups do not appreciably alter the affinity, thereby validating the inclusion of spermidine and spermine in this series.

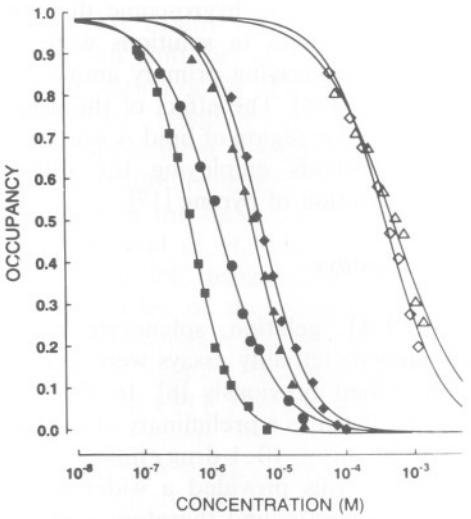

Fig. 2. Displacement of DC from lipid A by cationic drugs. Polymyxin B, solid circles; primaquine, solid triangles; pentamidine, solid squares; metformin, hollow triangles; phenformin, hollow diamonds; chlorhexidine, solid diamonds. DC concentration (45 μM); lipid A concentration (7 μM). Probe occupancy is calculated as $(F\text{-}F_o)/(F_{max}\text{-}F_o)$; F_o: fluorescence intensity of DC alone, F_{max}: fluorescence intensity of the DC-lipid A mixture at 0 μM drug concentration. Symbols represent means of duplicate data points and curves were fit using ALLFIT.

In the case of the phenothiazines for which apparent K_ds could not be obtained, static quenching constants derived from Stern-Volmer plots were used to compare their relative affinities for lipid A (Table 1). In these experiments, quenching experiments were also performed with DMPC, a neutral phospholipid which served as control. The magnitude of quenching of DPH incorporated into lipid A, as well as DMPC, is in the order trifluoperazine > chlorpromazine > promethazine (Table 1); however, the absolute values of the quenching constants are lower for DMPC.

Table 1

Drug	$K_{d(app)}$ (μM)	Relative potency	Stern-Volmer quenching constant (10^6 M^{-1})	
			Lipid A	DMPC
Polymyxin B	0.37	1.000	–	
Primaquine	0.65	0.322		–
Chloroquine	–	–		–
Trifluoperazine	–	–	0.192	0.081
Chlorpromazine	–	–	0.067	0.023
Promethazine	–	–	0.025	0.021
Metformin	71.8	0.004	–	
Phenformin	62.1	0.007		–
Chlorhexidine	0.87	0.462	–	
Pentamidine	0.12	2.651		–

K_d(app) was calculated by the Horovitz-Levitzki method. Relative potency is expressed as the ED_{50}(Polymyxin)/ED_{50}(Drug) ratio and was calculated from the displacement curves using ALLFIT.

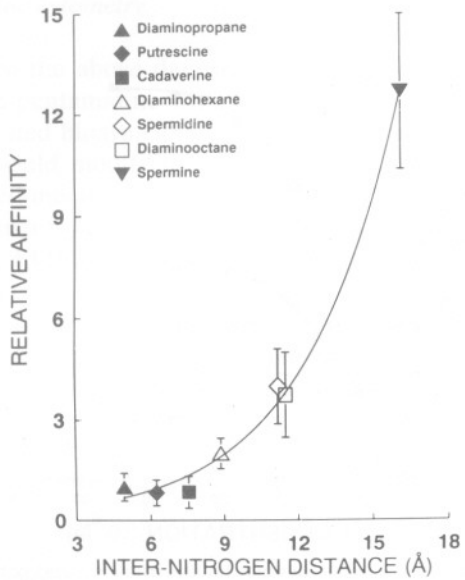

Fig. 3. DC displacement by diaminoalkanes and polyamines. Data points represent the mean of triplicate values. DC concentration (45 μM); lipid A concentration (7 μM). The affinity of the amines are expressed relative to diaminopropane (c3) as $ED_{50}(c3)/ED_{50}$(amine), calculated from displacement curves as described in legend to Fig. 1. Error bars represent the standard error of estimate (ALLFIT). An exponential curve was fit by nonlinear regression.

3.2. Antagonism in the Limulus assay

The effects of the drugs on lipid A-induced gelation of *Limulus* amebocyte lysate are represented in Table 2. With the exception of chloroquine, promethazine and metformin, all other compounds display lipid A antagonism to varying degrees. In the LAL experiments, drug-lipid A mixtures which did not elicit gelation were incubated with excess of lipid A (0.05 μg/ml); gelation occurred in all such samples indicating that the mechanism of inhibition of gelation by these compounds was by sequestration of free lipid A, and not due to direct inhibitory effects on the coagulation cascade of LAL by the drugs. Polymyxin B, pentamidine and chlorhexidine were also examined at 1:1 drug/lipid A molar ratios. In these experiments, gelation typically occurred at lipid A concentrations of one order of magnitude lower than that obtained with 10:1 molar ratio mixtures. The relative affinity of the drugs for lipid A (Table 1) roughly correspond to their inhibitory activity in the LAL assay (Table 2).

Among the drugs examined, pentamidine was particularly interesting because of its high affinity for lipid A and its consequent antagonism in the LAL assay, its widespread clinical use, and recent reports of protective effects of the drug in an animal model of endotoxic shock [21]. This compound was therefore further characterized in additional experiments as described below. Where possible, the effects of pentamidine were compared with those of polymyxin B.

Table 2
Limulus gelation activity of free lipid A and 10:1 molar ratio drug-lipid A mixtures

Compound	Lipid A concentration/ml				
	1 μg	100 ng	10 ng	1 ng	100 pg
Lipid A (LA)	+	+	+	+	–
LA-Polymyxin B	–	–	–	–	–
LA-Primaquine	–	–	–	–	–
LA-Chloroquine	+	+	+	+	–
LA-Trifluoperazine	–	–	–	–	–
LA-Chlorpromazine	+	+	+	–	–
LA-Promethazine	+	+	+	+	–
LA-Metformin	+	+	+	+	–
LA-Phenformin	+	+	+	–	–
LA-Chlorhexidine	–	–	–	–	–
LA-Pentamidine	–	–	–	–	–

3.3. Biological assays

The activity of lipid A in the splenocyte proliferation (Fig. 4A) and chick embryo lethality assays (Fig.

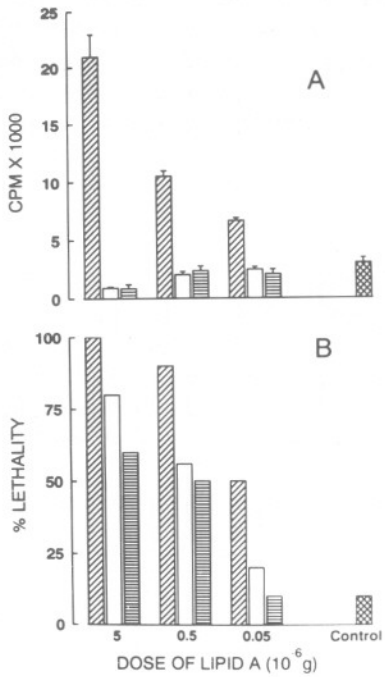

Fig. 4. (A) Mitogenic activity of free lipid A (hatched bars), and lipid A-pentamidine complexes (blank bars) prepared by incubating lipid A with 10 molar excess of pentamidine. ^{3}[H]Thymidine incorporation in $5 \cdot 10^6$ BALB/c murine splenocytes. Concentrations indicated correspond to that of lipid A for free lipid A and drug-lipid A complexes. Concentrations of free pentamidine (horizontal stripes) are identical to that present in drug-lipid A mixture. Cross-hatched bar represents RPMI control. Error bars correspond to standard deviations calculated on quadruplicate samples. (B) Lethal toxicity in 12-day-old chick embryos. Free lipid A, hatched bars; complexes prepared by incubating lipid A with 10 molar excess of pentamidine, blank bars; pentamidine alone at corresponding concentrations, horizontal stripes; saline control, cross-hatched bar. Percentages are calculated from batches of 5 embryos.

4B) is significantly attenuated by pentamidine. The concentrations of the drug used in the splenocyte proliferation experiments are non-toxic as shown by LDH release (data not shown). Furthermore, the mitogenic response to non-LPS stimuli (concanavalin A) is identical in the presence or absence of the drug (data not shown). The lethality observed in chick-embryos with the higher doses of pentamidine is to be expected in view of the susceptibility to a wide range of agents [22]; however, the toxicity elicited by the complex is significantly lower than that of free lipid A. These results are therefore indicative of sequestration of the bioactive form(s) of lipid A by pentamidine.

3.4. NMR

The addition of LPS to pentamidine resulted in a concentration-dependent line broadening of pentamidine resonances (Fig. 5) and the addition of excess of LPS resulted in complete disappearance of pentamidine signals, concomitant with the formation of macroscopic precipitates (data not shown). The resonances corresponding to the hydroxyethanesulfonate counterion of pentamidine is unaffected by LPS, serving as an internal control to indicate that the observed line broadening was not artifactual (increased sample viscosity etc.). 2D NOESY experiments were carried out in an effort to characterize the interactions in greater detail. In these experiments intermolecular NOEs were not observed under the experimental conditions used. However, the intramolecular NOE crosspeaks corresponding to the bis-amidine resonances were uniformly negative while the isethionate resonances showed positive NOEs (data not shown). These results are indicative of immobilization of pentamidine in the presence of LPS, and therefore imply binding of the drug to LPS. Generalized broadening of polymyxin B resonances upon binding to LPS has been reported elsewhere [23].

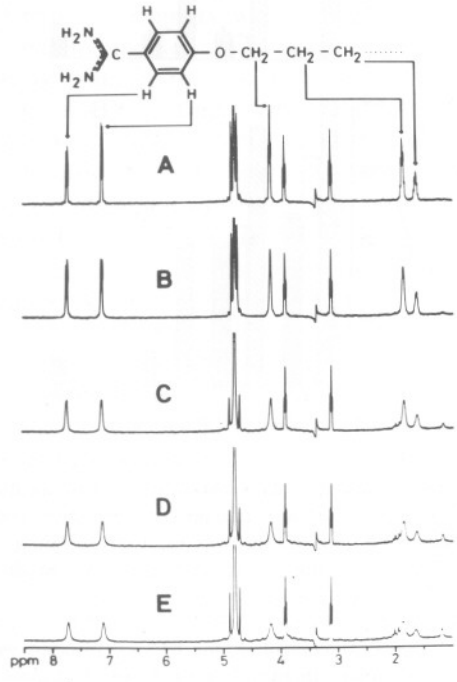

Fig. 5. 400 MHz ^{1}H-NMR spectrum of pentamidine in D_2O at 5 mM concentration at 25°C with the resonances assigned (a). Small aliquots of a stock suspension of *E. coli* 026:B6 LPS in D_2O were added successively. The final concentrations of LPS per ml are: (a) 0 mg; (b) 0.5 mg; (c) 1.0 mg; (d) 1.5 mg; (e) 2.0 mg.

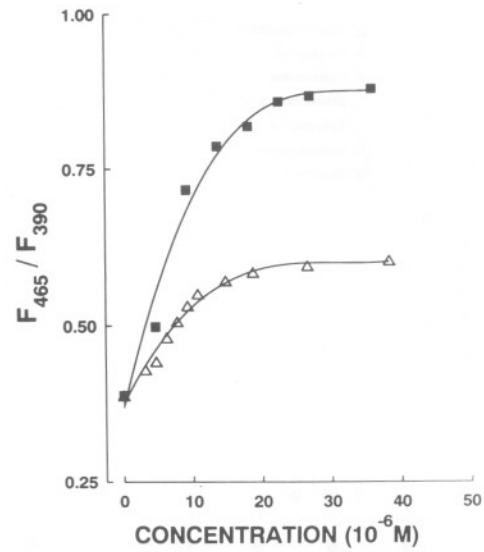

Fig. 6. The effect of pentamidine (hollow triangles) and polymyxin B (solid squares) on the acyl chain fluidity of lipid A as probed by the excimer fluorescence of pyrene. Excitation: 340 nm. The ratios of fluorescence intensities at 465 nm (excimer) and 390 nm (monomer) are represented as a function of drug concentration. Pyrene, 5 μM; Lipid A, 17.5 μM. In control experiments using the drugs in the absence of lipid A, no effect on the excimer/monomer ratio was observed.

3.5. Effect on the acyl chain fluidity of lipid A

Both pentamidine and polymyxin B increase the excimer/monomer ratio of pyrene incorporated in lipid A (Fig. 6) which is suggestive of fluidization of the acyl regions of lipid A. However, this interpretation assumes that the local concentration of pyrene remains constant. An alternative explanation would be a partitioning of the probe out of lipid A upon drug-binding which would increase local concentrations of pyrene thereby causing an artifactual increase in the excimer/monomer ratio. The latter interpretation is more likely since we observed quenching of DPH fluorescence in the presence of the drugs, presumably resulting from lateral phase separation of the lipid, partitioning out of DPH, and subsequent self-quenching of the probe (data not shown).

3.6. Flow cytometry

Since the above data are consistent with complexation of pentamidine with LPS/lipid A, manifested in diminished bioavailability, we sought to examine if the drug would modify the propensity of LPS to interact with cell membranes. The results of the flow cytometry experiments (Fig. 7) are indicative of decreased binding of FITC-LPS when presented to murine spleen cells as complexes with either pentamidine or polymyxin B. The formation of intensely fluorescent drug/FITC-LPS aggregates was responsible for the appearance of the small right-shifted accessory fluorescence peak in the case of cell samples incubated with mixtures of FITC-LPS and the drugs (Fig. 7). This was confirmed by fluorescence microscopy.

4. Discussion

Our earlier studies on the interaction of peptides with lipid A had suggested that basicity and hydrophobicity are important structural features in determining their endotoxin-binding properties [8,9]. This is not surprising given the amphiphilic and anionic nature of LPS and lipid A [1], which enable them to bind diverse cationic substances, among them metal cations, oligoamines and polyamines [24], cationic dyes [25], antibiotics [26], and basic proteins [27]. However, the binding of the peptides to lipid A appeared to be relatively independent of their amino acid sequence, suggesting that the interactions are not sensitive to the conformations of the peptides, and that basicity and hydrophobicity may be both necessary and sufficient. The experiments reported here were performed to examine whether such an inference could be extended to encompass small molecules; these results suggest that several basic and hydrophobic drugs, already in therapeutic use, bind lipid A and their affinities of interaction correlate roughly with their in vitro endotoxin-antagonistic properties.

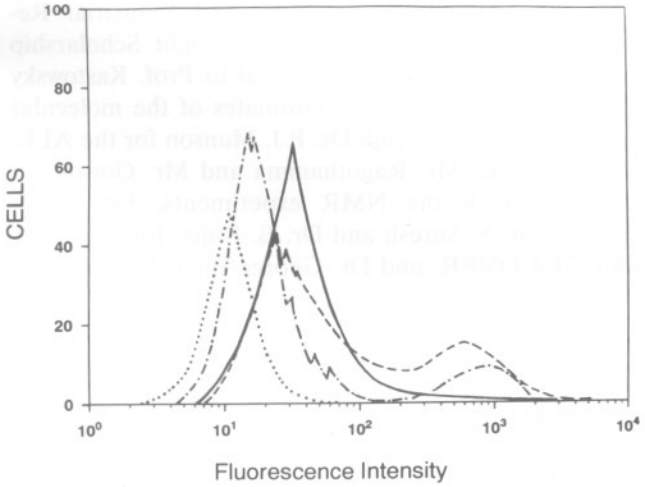

Fig. 7. The effect of complexation of FITC-LPS with pentamidine and polymyxin B on its binding to murine splenocytes analyzed by flow cytometry. Smoothed fluorescence intensity profiles of unlabelled cells (·····), cells incubated with free FITC-LPS (———), and with FITC-LPS preincubated with approx. 10 molar excess of pentamidine (— — —) or polymyxin B (------).

In the case of the phenothiazines, the quenching constants of the drugs for lipid A parallel that for DMPC (Table 1), suggesting that their differential affinities for lipid A may be a consequence of their varying hydrophobicity [28]; however, the absolute magnitudes of quenching are lower for DMPC, signifying that the anionic character of lipid A facilitates binding via electrostatic interactions. A comparison of the structures of chloroquine and primaquine suggests that the absence of primary amino groups in chloroquine may reduce its propensity for ionic interactions with the phosphates on lipid A. These considerations are merely suggestive of some qualitative structure-function relationships in these molecules, and clearly require a rigorous examination of larger families of compounds. It must be noted that the drug-binding experiments were performed at low ionic strength, and therefore the relative affinities are probably overestimated owing to the electrostatic double layer which is likely to exist at the lipid A-solvent interface. However, these empirical values may be used for purposes of comparison. It was not possible to examine the stability of the complexes or the affinities of interactions as a function of ionic strength or in the presence of detergents for two reasons: (a) the destabilization of the fluorescent probe-lipid A complex and (b) altered supra-molecular states of lipid A complicating the interpretation of results under these conditions.

The relative affinity and antagonistic activity of the bis-biguanide drug, chlorhexidine, is strikingly greater than the biguanides, suggesting that dicationic molecules may be more effective antagonists, perhaps as a consequence of simultaneous interactions with the phosphate groups on the backbone of lipid A. In the case of pentamidine, the presence of two strongly basic amidines which are protonated at physiological pH, separated by a distance that is commensurate with the inter-phosphate distance of lipid A rendered it particularly attractive, and perhaps not surprisingly, its affinity was found to be even greater than that of polymyxin B. The spectroscopic studies provide unequivocal evidence for direct interactions of pentamidine with both free lipid A and LPS and the resultant complexes display attenuated toxicity in biological assays.

A discussion of the binding of pentamidine and other dicationic molecules will have to take into consideration the supramolecular state of lipid A not only to explore the possible modes of interaction of these

molecules with the lipid, but also in view of the observation that its physical state can differentially modify the expression of toxicity [29], and the fact that endotoxicity cannot be ascribed to topologically defined 'toxophore' groups, but rather depend on the overall conformation and physical states of the toxin [30]. The experimental conditions used in this study (negligible divalent cation concentration, 25°C, i.e., below the phase transition temperature of lipid A) would predispose the lipid to exist as unilamellar structures [31] – an observation which has been corroborated in our laboratory by electron microscopy (unpublished data). Therefore, under these conditions, the drugs would not encounter monomeric toxin molecules, but interact with unilamellar aggregates which present a polyanionic surface lattice with an ordered, two-dimensional array of anionic phosphate groups. The experiments with diamino molecules with increasing end-to-end distances suggest that there may be an optimal distance between the cationic termini, possibly reflecting the nature of dispersion of the anionic sites on the lipid aggregates. It should be noted that the exponential relationship observed in the case of the diaminoalkanes and polyamines between the internitrogen distance and their affinity toward lipid A cannot continue indefinitely and must reach an extremum at some point. It would therefore be useful to examine higher homologs, and also analogs with alkyl or aromatic substituents which may permit the evaluation of possible hydrophobic or van der Waals effects. The dimensions of pentamidine may allow the terminal amidines to bind both phosphates of the same lipid A molecule, or, the drug could crosslink adjacent lipid A molecules. We have attempted to address this question by comparing the affinities of interaction of pentamidine with mono and diphosphoryl lipid A. The near-identical affinities would favor the cross-linking type interaction; however, this interpretation assumes that the physical states of the two forms of the toxin are comparable and conclusive evidence must await further studies.

Several aspects of pentamidine-lipid A/LPS interactions merit further attention. These experiments have not adequately addressed the effect of drug-binding on the physical state of lipid A or LPS. Although preliminary electron microscopic investigations show a complete disruption of the bilayer structure of lipid A with the formation of large aggregates upon drug-binding (unpublished data), the results are relatively uninformative, indicative only of gross alterations. A more detailed analysis using methods such as X-ray diffraction is likely to prove instructive. It would be useful to examine the mode of binding of dicationic molecules to lipid A in greater detail; experiments using lipid A dispersed in unilamellar vesicles are now in progress. Finally, although the results of the biological assays reported here are promising, these experiments have used free lipid A, an LPS substructure that organisms do not normally encounter. We elected to study lipid A because the heterogeneity and variability of LPS precludes quantitative analysis. We have now examined the effect of pentamidine at therapeutic concentrations on the induction of several cytokines by whole LPS in cultured human peripheral blood mononuclear cells and this will be the subject of a future publication.

These results provide some insight into the nature of drug-endotoxin interactions and may serve to increase our understanding of the desirable structural properties of potential inhibitors of endotoxicity. The identification of endotoxin antagonistic properties in a therapeutically well-characterized drug – pentamidine – may provide a point of departure in efforts directed at developing specific antagonists for endotoxin. The structure of pentamidine lends itself to several possible modifications which would be of interest: for instance, the terminal amidines may be replaced by guanidines; the latter, with its multiple H-bond donor atoms and favorable planar geometry have been implicated in the recognition of phosphates [32] and successfully exploited in the design of synthetic receptors for phosphodiesters [33]. An analysis of analogs with varying carbon-chain length [34] may help identify candidate molecules of optimal length. Derivatives with the linker alkane acylated may enable penetration and more effective disruption of the LPS superstructures. These possibilities are currently being addressed.

Acknowledgements

SD is a recipient of a Senior Research Fellowship from the Council for Scientific and Industrial Research, New Delhi. BB was a Fulbright Scholarship awardee. The authors are grateful to Prof. Kastowsky for providing the atomic coordinates of the molecular model of core-glycolipid, Dr. P.J. Munson for the ALLFIT programs, Mr. Ragothamma and Mr. Gowda for assistance with the NMR experiments, Dr. Sowdhamani, Mr. S. Suresh and Dr. B. Rajini for their help with DISCOVER, and Dr. George Babu for help with flow cytometry.

References

[1] Raetz, C.R.H. (1990) Annu. Rev. Biochem. 59, 129–170.
[2] Nathan, C.F. (1987) J. Clin. Invest. 79, 319–326.
[3] Hamil, R.J. and Maki, D.G. (1986) in Handbook of Endotoxin (Proctor, R.A., ed.), Vol. 4, pp. 55–126, Elsevier, Amsterdam.
[4] Johnston, J. (1991) J. NIH. Res. 3, 61–65.
[5] Galanos, C., Lüderitz, O., Rietschel, E.T., Westphal, O., Brade, H., Brade, L., Freudenberg, M., Schade, U., Imoto, M.,

Yoshimura, H., Kusumoto, S. and Shiba, T. (1985) Eur. J. Biochem. 148, 1–5.
[6] Morrison, D.C. and Jacobs, D.M. (1976) Immunochemistry 13, 813–818.
[7] Stokes, D.C., Shenep, J.L., Fishman, M., Hildner, W.K., Bysani, G.K. and Rufus, K. (1989) J. Infec. Dis. 160, 52–57.
[8] David, S.A., Mathan, V.I. and Balaram, P. (1992) Biochim. Biophys. Acta 1123, 269–274.
[9] David, S.A., Balaram, P. and Mathan, V.I. (1993) Med. Microbiol. Lett. 2, 42–47.
[10] Narayanan, R. and Balaram, P. (1976) Biochem. Biophys. Res. Commun. 70, 1122–1128.
[11] David, S.A., Balasubramanian, K.A., Mathan, V.I. and Balaram, P. (1992) Biochim. Biophys. Acta 1165, 147–152.
[12] Horovitz, A. and Levitzki, A. (1987) Proc. Natl. Acad. Sci. USA 84, 6654–6658.
[13] DeLean, A., Munson, P.J. and Rodbard, D. (1978) Am. J. Physiol. 235, E97–E108.
[14] Mellinger, T.J. and Keeler, C.E. (1963) Anal. Chem. 35, 554–558.
[15] Ma, J.Y.C., Ma, K.K.H. and Weber, K.C. (1985) J. Lipid Res. 26, 735–744.
[16] Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W. and Weigele, M. (1972) Science 178, 871–872.
[17] Antunes-Madeira, M.C., Almeida, L.M. and Madeira, V.M.C. (1990) Biochim. Biophys. Acta 1022, 110–114.
[18] Kastowsky, M., Sabisch, A., Gutberlet, T. and Bradaczek, H. (1991) Eur. J. Biochem. 197, 707–716.
[19] Lowe, P.R., Sansom, C.E., Schwalbe, C.H. and Stevens, M.F.G. (1989) J. Chem. Soc., Chem. Commun. 16, 1164–1165.
[20] Huse, Y. and Iitaka, Y. (1969) Acta. Cryst. B25, 498–509.
[21] Rosenthal, G.J., Craig, W.A., Corsini, E., Taylor, M. and Luster, M.I. (1992) Toxicol. Appl. Pharmacol. 112, 222–228.
[22] Finkelstein, R.A. (1964) Proc. Soc. Exp. Biol. Med. 115, 702–707.
[23] Kubesch, P., Boggs, J., Luciano, L., Maass, G. and Tummler, B. (1987) Biochemistry 26, 2139–2149.
[24] Galanos, C. and Lüderitz, O. (1975) Eur. J. Biochem. 54, 603–610.
[25] Ogawa, Y. and Kanoh, S. (1984) Microbiol. Immunol. 12, 1313–1323.
[26] Hancock, R.E.W., Farmer, S.W., Li, Z. and Poole, K. (1991) Antimicrob. Agents Chemother. 35, 1309–1314.
[27] Marra, M.N., Wilde, C.G., Griffith, J.E., Snable, J.L. and Scott, R.W. (1990) J. Immunol. 144, 662–666.
[28] Ford, J.M., Prozialeck, W.C. and Hait, W.N. (1989) Molec. Pharmacol. 35, 105–115.
[29] Galanos, C. and Lüderitz, O. (1984) in Handbook of Endotoxin (Rietschel, E.T., ed.), Vol. 1, pp. 46–58, Elsevier, Amsterdam.
[30] Lüderitz, O., Freudenberg, M.A., Galanos, C., Lehmann, V., Rietschel, E.T. and Shaw, D.H. (1982) in Current topics in membranes and transport (Razin, S. and Rottem, S., eds.), Vol. 17, pp. 79–151, Academic Press, New York.
[31] Seydel, U., Brandenburg, K., Koch, M.H.J. and Rietschel, E.T. (1989) Eur. J. Biochem. 186, 325–332.
[32] Calnan, B.J., Tidor, B., Biancalana, S., Hudson, D. and Frankel, A.D. (1991) Science 252, 1167–1171.
[33] Dixon, R.P., Geib, S.J. and Hamilton, A.D. (1992) J. Am. Chem. Soc. 114, 365–366.
[34] Cory, M., Tidwell, R.R. and Fairley, T.A. (1992) J. Med. Chem. 35, 431–438.