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Characterization of the interaction of lipid A and lipopolysaccharide with human serum albumin: implications for an endotoxin carrier function for albumin

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SUMMARY. The interactions of lipid A and lipopolysaccharide (LPS) with human serum albumin (HSA) were examined using fluorescence methods. Lipid A binds HSA with a stoichiometry of 2:1 with dissociation constants of 1.0 μM and 6.0 μM for the high- and low-affinity interactions, respectively. Lipid A displaces HSA-bound dansylsarcosine competitively, but not HSA-bound warfarin, suggesting that domain III-A, and not domain II-A, is a lipid A binding site. Domain I does not contribute a site for lipid A. Based on these data, and the structural similarity between subdomains III-A and III-B, it is proposed that these two regions of HSA represent the high- and low-affinity sites of interaction of lipid A. Whole LPS also binds HSA, displacing dansylsarcosine, and its lipid A moiety appears to be the interaction site. However, there are differences between LPS and free lipid A. Polymyxin B forms ternary complexes with LPS bound to HSA, suggesting that the regions on LPS recognized by HSA and polymyxin B are different. The observed affinity of lipid A for HSA and mass action effects due to its abundance in the circulation would imply a major LPS carrier function for HSA.

Endotoxins, or lipopolysaccharides, are important structural constituents of the outer membrane of Gram-negative bacteria.¹ These amphiphilic macromolecules elicit a variety of biological effects in susceptible hosts² and have been implicated in the pathogenesis of septic shock, an important cause of mortality worldwide.³ Knowledge of the nature of interactions of endotoxin with serum components would help address the questions of how these potentially deleterious molecules are processed and distributed in vivo and how they ultimately reach their target tissues and mediate their effects.

Abbreviations: HSA – human serum albumin; LPS – lipopolysaccharide; FITC-LPS – fluorescein isothiocyanate-labelled lipopolysaccharide; IAEDANS – 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid.

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Because of their exterior location on the bacterial membrane, they are readily accessible for interaction with serum constituents. Indeed, several normal serum components other than LPS-specific antibodies such as high density lipoproteins,^{4,5} complement components,^{6,7} lysozyme,^{8,9} albumins,¹⁰ and elicited plasma proteins, important among which, lipopolysaccharide-binding protein,^{11,12} are known to bind LPS. However, the structural heterogeneity of LPS¹³ has been a major impediment to detailed analyses of these interactions. We have therefore used lipid A, a structurally well-characterized and considerably less heterogeneous substructure of LPS which elicits almost the entire spectrum of biological activities of native LPS¹⁴ as a model endotoxin. We report in this paper the characterization of the interaction of lipid A with human serum albumin (HSA) which was facilitated by the availability of site-specific probes for HSA, the presence of a single tryptophan and free cysteine thiol

group serving as unique spectroscopic handles, and the recent elucidation of its high resolution atomic structure. In extending these findings qualitatively to whole LPS, we find differences between lipid A and LPS and relate these observations to the known physicochemical differences between them.

MATERIALS AND METHODS

Dansylsarcosine (piperazinium salt), warfarin sodium, LPS and fluorescein isothiocyanate-conjugated LPS from *Escherichia coli* 026:B6 (FITC-LPS), essentially fatty acid-free human serum albumin (HSA), and dansyl chloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Hexacyl-type diphosphoryl lipid A from *E. coli* k12-D31m4 was from List Biologicals (Campbell, CA, USA) and was solubilized in 0.1% triethylamine by vigorous vortexing above its phase transition temperature (60°C). 5-(((2-iodoacetyl) amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS) was from Molecular Probes (Eugene, OR, USA). Fluorescence experiments were performed using a Shimadzu RF-5000 spectrofluorimeter. All experiments were carried out in 10 mM Tris-HCl buffer, pH 7.4 at 25°C. Additions of lipid A to HSA did not result in appreciable pH changes. All experiments reported here were performed under steady-state conditions. Intensity and wavelength shifts occurred within a few seconds following the addition of reagent. An equilibration period of 5 min was allowed after each addition of reagent.

Intrinsic fluorescence of HSA

Small aliquots of lipid A stock suspension were successively added to HSA (4.43 μ M) in buffer. An excitation wavelength of 275 nm (5 nm bandpass) was used. Emission spectra were obtained by scanning from 290 to 425 nm (3 nm bandpasses), and intensities were recorded at wavelengths of maximal emission (335 nm in the absence of lipid A, gradually blue-shifting to 321 nm at saturating lipid A concentrations). Steady state emission polarization values were obtained at 10 nm bandpasses as described earlier.¹⁵

Fluorescent probe displacement

Dansylsarcosine and warfarin were used as site-specific reporters to probe ligand binding to domains III-A (Site II) and II-A (Site I) respectively.¹⁶ Aliquots of stock solutions of these probes were added to HSA in buffer (4.43 μ M) such that their final concentrations were typically 7.5–15 μ M. Excitation and emission wavelengths for dansylsarcosine and warfarin were 350 nm/475 nm, and 320 nm/385 nm, respectively with bandpasses of 5 nm for both monochromators. Free solubilized lipid A has negligible extinction coefficients at these wavelengths and thus corrections for filter effects were unnecessary. Aliquots of lipid A were then added to the HSA-probe mixtures, and the intensities of probe emission were analyzed as a function of lipid A concen-

tration. Bound fluorescent probe concentrations at each lipid A concentration were computed from values of saturating fluorescence (maximal emission when the probe is completely bound to HSA). These values were derived from double-reciprocal plots constructed for either probe by titrating HSA against a fixed concentration of probe. Similar experiments were also performed using indirect excitation of the probes via energy transfer from the lone tryptophan of HSA; an excitation wavelength of 280 nm was used in these experiments.

Dansylation of HSA

The single free thiol of HSA (cysteine-34) was dansylated to serve as a probe for lipid A binding to domain I. HSA (10 mg) was dissolved in 1 ml 200 mM Tris-HCl, pH 8.0 to which was added a 1-molar excess of IAE-DANS. This mixture was incubated at ambient temperature in the dark and the reaction terminated at 20 min by adding a 50-molar excess of cysteine. Dansylated HSA was subsequently purified by gel-filtration on a Sephadex G-10 column, and lyophilized. The stoichiometry of dansyl:HSA was determined by spectrophotometry to be 1.12:1 (dansyl – extinction coefficient at 330 nm = 3,300 M⁻¹; HSA – extinction coefficient $E_{278}^{0.1\%}$ = 0.531¹⁷). The emission characteristics of dansyl-HSA were examined at 325 nm (tryptophanyl) and 465 nm (dansyl) by both direct excitation (340 nm) and indirect excitation via the lone tryptophan of HSA (280 nm). Steady state polarization of dansyl emission was obtained at 10 nm bandpasses as described earlier.¹⁵

Determination of binding parameters

Dissociation constants of lipid A for HSA were obtained by several independent methods. Scatchard analyses of changes in the intrinsic fluorescence parameters of HSA (tryptophanyl emission intensities and wavelength of maximal emission) were performed as described elsewhere.¹⁸ Probe displacement data were analyzed by the Horovitz-Levitzki method¹⁹ as described earlier,¹⁸ and by LIGAND, a nonlinear least-squares curve fitting algorithm.²⁰

Experiments with LPS

Warfarin and dansylsarcosine displacement experiments were performed as described above. Binding affinities were not computed owing to the considerable heterogeneity of the LPS preparations and probe displacements were expressed as a function of mass (μ g) of LPS added. The effect of LPS on the intrinsic fluorescence could not be reliably determined because of the presence of protein contaminants in LPS. Evidence for the complexation of whole LPS with HSA was also obtained by titrating FITC-LPS against a fixed concentration of dansyl-HSA; the spectral overlap of the dansyl/FITC (but not of Trp-214/FITC) donor-acceptor pair is of sufficient magnitude to monitor the interaction by means of energy transfer between the fluorophores.

Table 1.

Parameter	K _d (1)	K _d (2)	Method
Tryptophanyl emission intensity changes	1.998 μ M	6.389 μ M	Scatchard
Wavelength of maximal tryptophanyl emission	—	6.238 μ M	Scatchard
Dansylsarcosine displacement	0.954 μ M	—	LIGAND
Dansylsarcosine displacement	1.276 μ M	—	Horovitz-Levitzki

Formation of ternary complexes with polymyxin B

We sought to examine if polymyxin B could complex with lipid A or LPS bound to HSA since this could provide information on whether the sites on the toxin recognized by HSA and polymyxin B are different. A scheme using successive fluorescence energy transfer from Trp-214 on HSA to polymyxin B and LPS labelled with suitable fluorophores was used. Polymyxin B was dansylated according to a procedure described by Ohno et al.²¹ The FITC label on LPS was the secondary acceptor fluorophore. Spectral overlaps of the Trp-214/dansyl and dansyl/FITC donor-acceptor pairs enabled energy transfer from Trp-214 to FITC via the dansyl fluorophore. To 10 μ M of HSA in buffer 50 μ g FITC-LPS was added; successive aliquots of a stock solution of dansyl-polymyxin B were then added to this mixture. Excitation was at 275 nm and the emission spectra of the samples were recorded from 300–600 nm. As control experiments, fluorescence titrations of dansyl-polymyxin B with HSA in the absence of FITC-LPS, and of FITC-LPS with dansyl-polymyxin B in the absence of HSA were performed. Neither polymyxin B nor dansylated polymyxin B binds to HSA. The

scheme described above could not be used in the case of lipid A which lacks derivatizable groups, precluding labelling with a secondary acceptor fluorophore. In none of these experiments attempts were made to derive inter-fluorophore distances from energy transfer data since the site(s) and stoichiometry of labelling of LPS with FITC are unknown.

RESULTS

Intrinsic fluorescence of HSA

The addition of lipid A to HSA results in blue-shifting of the maximal emission wavelength of the lone tryptophan (Trp-214) of HSA (Fig. 1), saturating at a lipid A:HSA molar ratio of 2:1 (Fig. 2). The emission intensity of Trp-214 is distinctly biphasic (Fig. 1), manifesting initially in quenching which is maximal at 1 equivalent of lipid A, and from 1 to 2 equivalents of lipid A, in emission enhancement (Fig. 2). These results are indicative of a lipid A:HSA stoichiometry of 2:1, and the biphasic behavior of Trp-214 intensity is suggestive of sequential occupancy. Dissociation constants of lipid A for HSA obtained by Scatchard treatment of these data are presented in Table 1. The steady-state emission polarization value of Trp-214 in the absence of lipid A is 0.154 ± 0.003 , and remains essentially unchanged upon lipid A addition, signifying that Trp-214 does not experience hindered rotational mobility in the presence of lipid A.

Probe displacement

The addition of HSA to either dansylsarcosine or warfarin results in considerable enhancements in emission intensities of the probes as expected, consistent with the binding of the probes to HSA.²² The addition of lipid A to HSA-bound dansylsarcosine results in quenching of fluorescence (Fig. 3), while lipid A, by itself, is without effect on free dansylsarcosine. The addition of dansylsarcosine to HSA pre-incubated with a 10-molar excess of lipid A does not result in enhancement of the probe fluorescence. These results are indicative of competitive displacement of dansylsarcosine from HSA by lipid A. Dissociation constants for lipid A were obtained by analysing the displacement data by fitting a one-site competitive model using LIGAND, and by Horovitz-Levitzki analysis. Both methods allow the K_ds of the probe as well as the displacing ligand to be estimated.

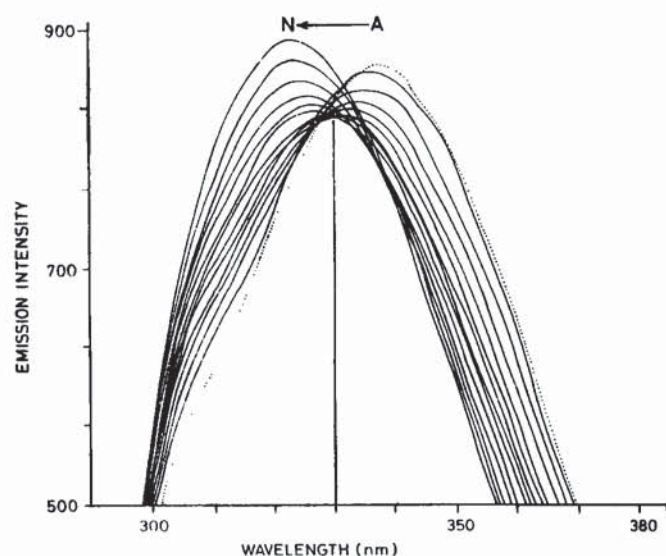


Fig. 1 — Effect of lipid A on tryptophanyl emission spectra of HSA. Excitation: 275 nm. [HSA]: 4.4 μ M. Spectrum A (dotted line), HSA in the absence of lipid A. Spectra B–N: 0.23 μ M aliquots of lipid A were incrementally added, equilibrated for 5 min, and spectra recorded. The HSA:lipid A molar ratio at which spectrum N was recorded was 1:1.47.

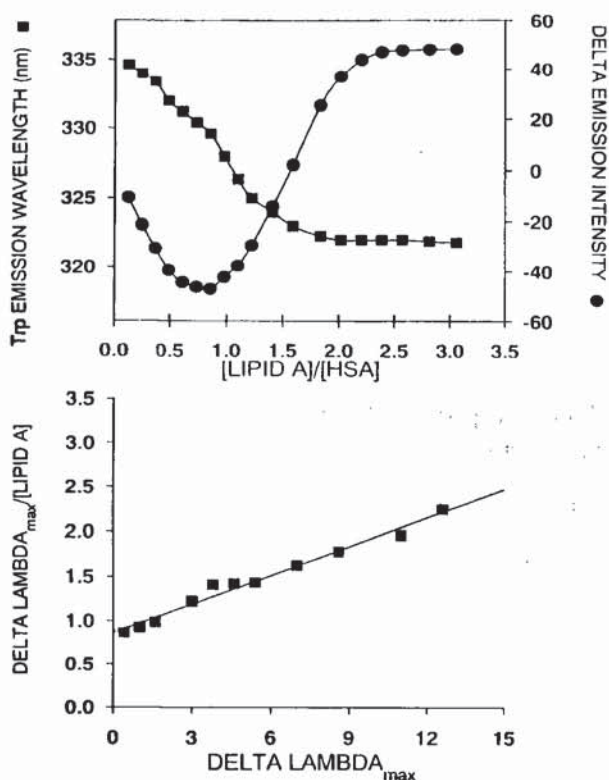


Fig. 2 — Top: Blue shift and biphasic intensity changes of HSA fluorescence plotted as a function of lipid A:HSA molar ratio. Wavelength of maximal emission of Trp-214 (squares); emission intensity in arbitrary units (circles). [HSA]: 4.43 μM . Excitation: 275 nm. Bottom: Scatchard-type plot of lipid A-induced changes in maximal emission wavelength of Trp-214. The ratio of the modulus of change in emission wavelength to lipid A concentration is plotted as a function of the absolute value of the wavelength shift. An apparent K_d of 6.24 μM , represented by the slope of the fitted line was obtained.

The binding constants for lipid A were 0.95 μM and 1.27 μM (Table 1), and that of the probe were 6.11 μM and 7.16 μM , as computed by the two methods respectively. The latter values are in close agreement to those reported in the literature (6 μM).²²

Titration of lipid A with HSA-bound warfarin shows a biphasic response, with fluorescence enhancement saturating at approximately 2 equivalents of lipid A, followed by quenching at higher lipid A concentrations (Fig. 3). Control experiments as described above verified that lipid A was without effect on free probe, and lipid A sequestered by polymyxin B did not alter the fluorescence of HSA-bound probe. The enhancement of warfarin emission in the initial phase could represent increased probe binding as has been reported earlier,²³ or may be a consequence of lipid A-induced local conformational changes in HSA resulting in altered micro-environment of the bound probe. The quenching occurring at higher lipid A concentrations, may likewise, mirror conformational perturbations, or may be indicative of probe displacement. In order to further examine these possibilities, these experiments were also performed with indirect excitation of the bound probes

via Trp-214 of HSA, the results of which are shown in Figure 4.

The relative efficiency of energy transfer from Trp-214 of HSA to bound dansylsarcosine decreases with increasing lipid A concentration (Fig. 4) which, taken together with the above results is consistent with probe displacement. However, the emission intensity changes of bound warfarin changes only in the range of $\pm 1.5\%$ (Fig. 4), ruling out displacement.

In order to examine if domain I-A could be contributory to lipid A binding, we used HSA with the free thiol of Cys-34 dansylated. The addition of lipid A to dansylated HSA is without effect on (a) dansyl emission intensity, (b) steady-state dansyl emission polarization and (c) efficiency of energy transfer from Trp-214 to dansyl (data not shown) indicating that lipid A does not bind to this region.

LPS-HSA interactions

The addition of LPS labelled with FITC to dansylated HSA results in incremental quenching of dansyl emission which is coupled with FITC intensity enhancement; these changes are reversed upon adding

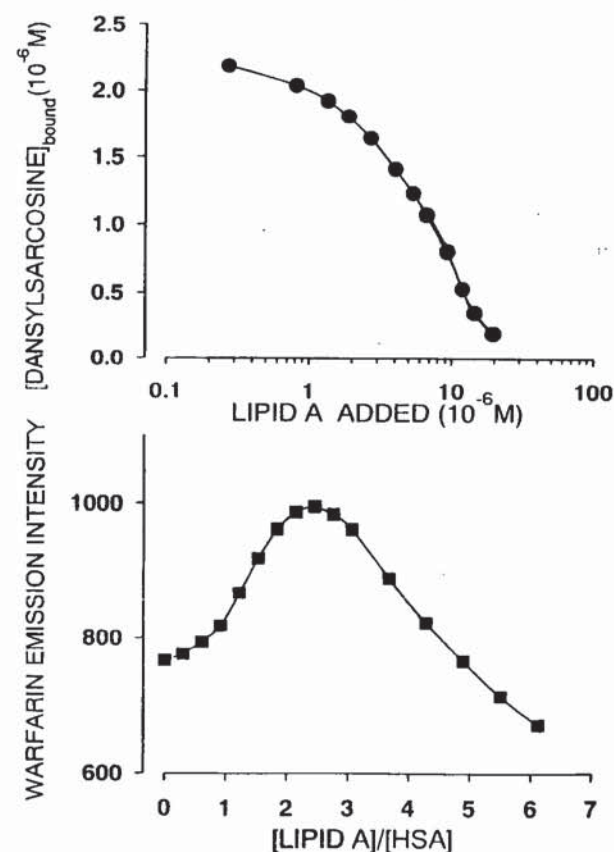


Fig. 3 — Effect of lipid A on fluorescent probes bound to HSA. Dansylsarcosine displacement (top) and effect on warfarin emission (bottom) as probed by direct excitation of probe. Bound dansylsarcosine concentrations (top) were determined as described in the text. HSA concentration was 4.43 μM in both experiments. Dansylsarcosine (top) concentration: 8.35 μM . Warfarin concentration (bottom): 8.3 μM .

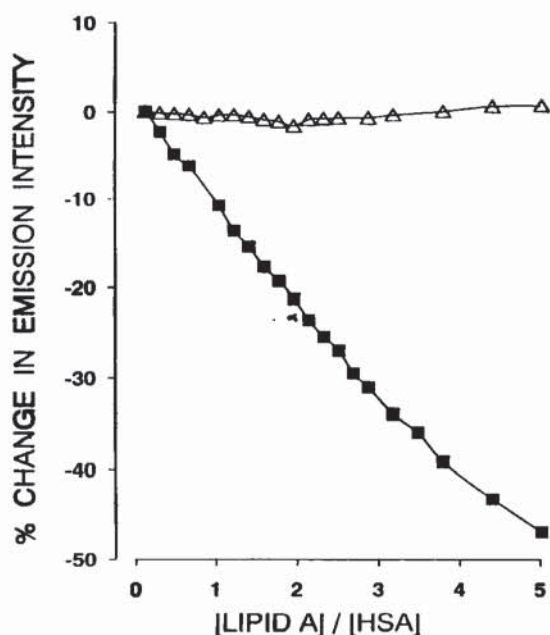


Fig. 4 — Lipid A induced changes in emission intensities of warfarin (hollow triangles) and dansylsarcosine (solid squares) bound to HSA as measured by indirect excitation and energy transfer from Trp-214. Excitation: 280 nm. [HSA]: 4.43 μ M. [Warfarin]: 8.5 μ M; [Dansylsarcosine]: 8.5 μ M.

either excess unlabelled LPS or lipid A (data not shown). Taken together, these results suggest that whole LPS also binds to HSA, and that the lipid A moiety of LPS represents the interaction site on the toxin molecule. LPS displaces dansylsarcosine from HSA, but at rather high concentrations (ED_{50} = approx. 250 μ g/ml), and unlike lipid A, does not enhance HSA-bound warfarin fluorescence at low concentrations, but quenches it in a mass-dependent manner (data not shown). Dansyl-polymyxin B forms ternary complexes with FITC-LPS bound to HSA as manifested by progressive quenching of HSA fluorescence which is paralleled by intensity enhancements of both dansyl and FITC fluorescence (Fig. 5) upon addition of dansyl-polymyxin B. These changes are indicative of energy transfer from Trp-214 to FITC via dansyl, since in control experiments, dansyl-polymyxin B quenches FITC-LPS in the absence of HSA (data not shown).

DISCUSSION

The highly conserved multi-domain structure of the serum albumins enable these molecules to serve as carrier proteins for a variety of chemically dissimilar molecules including otherwise insoluble ligands such as fatty acids and bilirubin.²⁴ Although the majority of these ligands are small anionic hydrophobic molecules, larger amphiphilic structures such as phospholipids have also been shown to bind albumin.²⁵ Indeed, indirect evidence of complexation of lipid A and LPS by albumin

already exists in the literature. Before it was established that satisfactory aqueous solubilization of lipid A could be achieved using triethylamine,²⁶ albumin had been used to disaggregate and solubilize lipid A.²⁷ Furthermore, it has been shown using whole LPS labelled with a photoactivable, radioiodinated, crosslinking probe that serum albumin is a major LPS-binding factor.¹⁰

The results of this study establish that two molecules of lipid A bind to HSA with apparent dissociation constants for the two sites corresponding to 1 μ M and 6 μ M, respectively. The use of site-specific probes in conjunction with the spectroscopic studies permit some conclusions with regard to the sites of lipid A interaction. The two important ligand binding sites, originally described as sites I and II by Sudlow et al.²² have been extensively characterized.^{16,23,28-30} Phenylbutazone and warfarin are typical of the ligands which interact with site I, and diazepam, 3'-azido-3'-deoxythymidine (AZT), and dansylsarcosine are examples of site II binding molecules. Sites I and II correspond structurally to domains II-A and III-A, respectively, according to the assignment based on the recently published high-resolution crystal structure of HSA,³¹ and the latter terminology will be used hereafter in discussing the binding sites of lipid A.

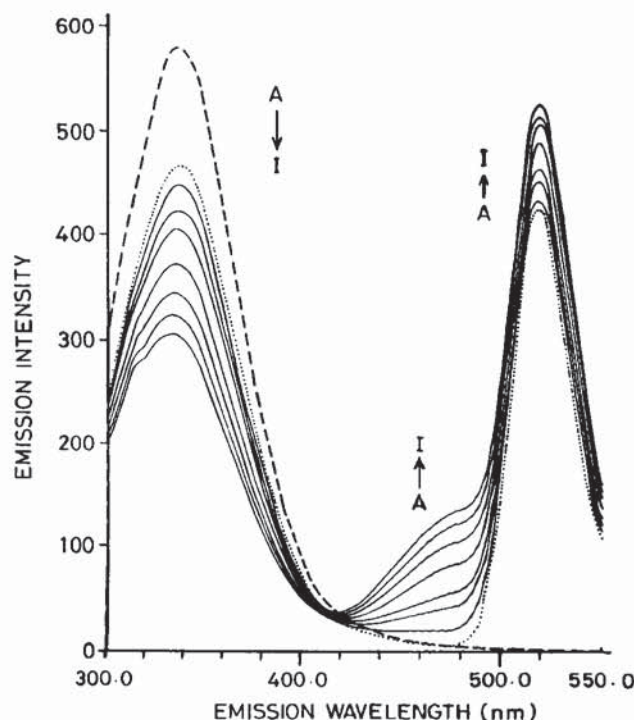


Fig. 5 — Non-competitive binding of dansylated polymyxin B to FITC-LPS complexed to HSA; demonstration of formation of ternary complexes. HSA (10 μ M) was preincubated with FITC-LPS (50 μ g) in buffer. Small aliquots of dansylpolymyxin were added, equilibrated for 5 min, and spectra recorded with excitation at 275 nm. Spectrum A (dashed line), HSA alone in buffer. Spectrum B (dotted line), HSA complexed to FITC-LPS in the absence of dansylpolymyxin. Spectra C-I: 1.2 μ M aliquots of dansylpolymyxin added sequentially. The peaks at 470 nm and 525 nm correspond to dansyl and FITC emission, respectively.

It is clear from the experiments using dansylated HSA that domain I does not constitute a site for lipid A, perhaps not a surprising finding given the unique structural disposition of this region.³¹ Although the warfarin experiments (Fig. 4) are suggestive of non-occupancy of domain II-A by lipid A, the fluorescence changes of the lone tryptophan (Trp-214), an important structural component of the II-A ligand binding region,³¹ may at first sight seem to be indicative of involvement of this site. However, steady-state emission polarization of tryptophan is unaltered in the presence of lipid A. It is recognized that ligands which bind to domain III can induce conformational changes in domain II because of a shared interface region between the two regions as is apparent in the crystal structure of HSA.³¹ Furthermore, Trp-214 also participates in the formation of the interface between the two domains.³¹ In the light of these structural considerations, we interpret the changes in Trp-214 and warfarin fluorescence to be the result of local conformational changes in domain II, as a consequence of lipid A binding to domain III. This would also be consistent with earlier reports on altered ligand binding to domain II-A upon domain III-A occupancy,²³ and the dependency of warfarin binding on the conformational state of albumin.³²

Since lipid A interacts with neither domain I nor II, it would be expected that domain III represents the site of interaction. It is evident from the dansylsarcosine displacement data that domain III-A is at least one of the sites, but does not resolve whether one or both sites are represented by this subdomain. Based on the above data, the estimated dimensions of lipid A,³³ and the fact that the subdomains A and B share a common structural motif,³¹ we propose that regions III-A and III-B represent the high and the low affinity sites of lipid A binding, respectively. The availability of crystal structure coordinates of HSA should enable this proposition to be examined by molecular modelling. It may be mentioned that this region contains several basic and hydrophobic residues³⁴ and it is likely that the binding of lipid A involves both electrostatic and hydrophobic interactions as is the case for several other lipid A-binding molecules.^{9,15,35}

Although whole LPS also binds to HSA through its lipid A moiety, there are differences between free lipid A and LPS with respect to their interactions with HSA. The reversal of energy transfer from dansylated HSA to FITC-LPS upon addition of lipid A, and the displacement of dansylsarcosine by LPS suggest that domain III-A is the recognition site. However, these data do not unequivocally delineate the stoichiometry of the HSA-LPS complex. Differential interactions which are apparently dependent on the chemotype structure of LPS have been noted with other circulatory proteins of mammalian origin such as lysozyme,^{36,37} which are thought to be a consequence of the different supramolecular states of LPS chemotypes. The smooth-form LPS containing the O-specific polysaccharide units tend to exist as closed lamellar structures which allow a limited exposure of the hydrophobic domains at the surface of the

molecular aggregates, while lipid A can exist in several non-lamellar forms³⁸ under experimental conditions similar to those employed in this study. Steric differences between lipid A and LPS may also be of importance, the latter possessing a rigid and bulky anionic core oligosaccharide region³⁹ as well as an extensive branched structure comprising of repeating polysaccharide units. Furthermore, recent theoretical calculations have shown that ionization states at physiologic pH of free lipid A and LPS (rough-mutant form) are considerably different,⁴⁰ which are likely to influence their solubilities. This is of importance since HSA can be expected only to interact with soluble lipid A or LPS molecules below their critical micellar concentrations. Although on theoretical grounds, one would predict the solubility of whole LPS to be higher than that of free lipid A, most preparations of LPS such as the one used in the present study contain substantial amounts of divalent (Ca^{2+} , Mg^{2+}) and organic (pyridinium) cations which greatly limit its solubility. Indeed, the sample of LPS we used yielded a highly turbid suspension in its concentrated form, while lipid A in the presence of triethylamine was an optically clear solution. Further studies using a series of LPS chemotypes obtained in salt forms of uniform aqueous solubilities are essential to address this issue.

Finally, it would be appropriate to comment on the biological relevance of these findings. The considerable affinity toward lipid A, and mass action effects by virtue of HSA being the most abundant serum protein would imply a major role as a carrier molecule for endotoxins. The existence of a cell surface receptor for albumin⁴¹ may point to a role for HSA in the delivery of LPS to the various target tissues. It is of interest that lipid A complexed with albumin exhibits the full spectrum of biological activities,²⁷ in this respect being similar to other serum factors which behave as LPS opsonins such as the Septins^{42,43} and lipopolysaccharide binding factor,^{11,12,44} while other serum proteins such as lysozyme or bactericidal/permeability increasing protein⁴⁵ can either partially or completely neutralize endotoxin activity. This may suggest that these LPS-binding proteins may recognize disparate regions on the endotoxin, and consequentially, present parts of the toxin molecule which are either toxically active or quiescent, to target cells. A unique 'toxophore' (or toxophores) on the lipid A/LPS molecule has not yet been identified. It is of relevance in this regard to note that the ability of polymyxin B to form ternary complexes with LPS bound to macromolecular ligands correlates with the retention of toxicity in the LPS-protein complexes. Both HSA (present study) and lysozyme³⁶ which either do not, or only partially modify the activity of complexed LPS permit noncompetitive interactions with polymyxin B and the consequent formation of ternary complexes, whereas proteins which neutralize endotoxin activity such as the *Limulus* anti-LPS factor,⁴⁶ or the HA-1A monoclonal antibody to lipid A⁴⁷ bind to LPS in a competitive manner with respect to polymyxin B. The elucidation of the structural basis of this phe-

nomenon would be of value in understanding mechanisms of endotoxin antagonism.

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