

Investigation into the interaction of recombinant human serum albumin with Re-lipopolysaccharide and lipid A

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The interaction of bacterial endotoxins, deep rough mutant lipopolysaccharide LPS Re and the 'endotoxic principle' lipid A, with recombinant human serum albumin (rHSA) was investigated with a variety of physical techniques and biological assays. With Fourier-transform infrared spectroscopy and differential scanning calorimetry, the influence of albumin on the acyl chain melting behavior of the endotoxins was measured. Also, the effect on the functional groups of the endotoxins, in particular with respect to their orientation, was studied, including competition experiments with polymyxin B. Furthermore, the influence of endotoxin binding to rHSA on the protein's secondary structure was investigated. The results indicate a non-electrostatic binding with no change of the backbone orientation of LPS and only a slight change of the secondary structure of rHSA. Correspondingly, the amount of charge neutralization of the endotoxins due to rHSA measured by the electrophoretic mobility exhibited only a slight reduction of the surface potential. From these measurements and isothermal titration calorimetry, the lipid:protein binding stoichiometry was estimated to [LPS]:[rHSA], 10:1 molar. The determination of the aggregate structure of the endotoxins by X-ray small-angle scattering exhibited a complex change of a cubic into a non-lamellar structure. No influence of rHSA on endotoxin intercalation into phospholipid liposomes induced by lipopolysaccharide-binding protein could be detected by fluorescence resonance energy transfer. Finally, the LPS-induced cytokine production of human mononuclear cells was only slightly increased at high molar rHSA excess, while the coagulation of amebocyte lysate in the *Limulus* test yielded a complex change due to rHSA binding of LPS.

INTRODUCTION

The mechanism of interaction of endotoxins with different target cell structures are still largely unknown, and only limited data are available about the detailed mode of binding of endotoxins to various endogenous proteins,

which are important with regard to invading micro-organisms and the transport and neutralization of free endotoxin. Many serum proteins, such as human serum albumin, are known to bind to endotoxin, but the function of this binding and the structural details are still unclear.^{1,2}

One of the group of these proteins is human serum albumin (HSA). HSA is one of the major plasma proteins (approximately 60% of the total). It consists of a single non-glycosylated polypeptide chain of 585 amino acids with a molecular weight of 66.5 kDa. HSA is a heart-shaped molecule, comprised of three domains; it is produced in the liver and is largely responsible for maintaining the normal osmolarity of the bloodstream, but also functions as a carrier for numerous small molecules,³ such as fatty acids, bilirubin, many drugs, and also certain hormones, when

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their specific binding globulins are saturated. Low serum levels occur in protein malnutrition, active inflammation and serious hepatic and renal disease.⁴

Knowledge of the nature of interactions of endotoxin with serum components would help to answer questions concerning the ways in which these potentially deleterious molecules are processed and distributed *in vivo* and, ultimately, reach their target and mediate their effects. Because they are located on the exterior of the bacterial membrane, they are readily accessible for interaction with serum constituents. It is likely that the binding of lipid A to albumin involves both electrostatic and hydrophobic interactions as is also the case for several other lipid A-binding molecules, although complete LPS also binds to human serum albumin through its lipid A moiety.⁵ There seems to be only a low level of specific binding of human serum albumin to endotoxin with unspecific binding at high protein concentration.⁶

The existence of a cell surface receptor for albumin⁷ may point to a role for albumin in the transport of LPS to the various target tissues. The role of HSA in LPS immunostimulatory activity is unclear. For example, it has been found that the rate of LPS-induced TNF- α production in peritoneal mouse macrophages is increased in the presence of pure albumin compared to serum or serum-free systems, from which an enhancing effect of albumin was deduced. Others observed TNF- α production induced by albumin alone, which was attributed to a contaminant from immunoglobulin.⁸ Also, high interleukin-6 (IL-6) inducing capacity in mouse macrophages was found using a commercial bovine serum albumin (BSA) preparation, which was not contaminated with endotoxins, in particular in the highly aggregated fractions.⁹ So far, the function of albumin seems to be completely different from those of other serum proteins such as lysozyme, lactoferrin or bactericidal/permeability increasing protein, which either partially or completely neutralize endotoxin activity.⁵ Trace amounts of endotoxin (LPS) are assumed to contaminate commercially available fetal bovine serum (FBS) for tissue or cell culture during the manufacturing process. The results of Kirikae *et al.* and Pool *et al.* show that many lots of FBS contain significant levels of LPS, which must affect cell culture, they noticed that pyrogen-free human serum albumin does not exist.^{10,11}

The aim of this study was the detailed biophysical characterization of the binding between endotoxin and human serum albumin. For this, we have taken recombinant human serum albumin (rHSA) to avoid any influence due to contamination.

MATERIALS AND METHODS

Lipids and reagents

Lipopolysaccharide from the deep rough mutant Re *Escherichia coli* (strain: F515) was extracted by the phe-

nol/chloroform/petrol ether method from bacteria grown at 37°C, purified, and lyophilized. Free lipid A was isolated by acetate buffer treatment of LPS F515. After isolation, the resulting lipid A was purified and converted to its triethylamine salt. The known chemical structure of lipid A from LPS F515 was checked by the analysis of the amount of glucosamine, total and organic phosphate, and the distribution of the fatty acid residues applying standard procedures. The amount of 2-keto-3-deoxyoctonate (Kdo) never exceeded 5%.

The formulation of the utilized recombinant human serum albumin (rHSA) was: rHSA, 12.5 g; N-acetyl-DL-tryptophan, 246.3 mg; sodium caprylate, 166.2 mg; and sodium chloride, 155 mg, pH 6.4–7.4.

The rHSA was tested for endotoxin contamination by the *Limulus* (LAL)-assay and passed the test with ≤ 0.3 EU/ml.

Sample preparation

The lipid samples were usually prepared as aqueous dispersions at high buffer content, *i.e.* above 90% using 20 mM HEPES for the physical techniques, and in embryo-transfer water (Sigma) at the respective concentrations in the biological tests. For this, the lipids were suspended directly in buffer, sonicated, temperature-cycled several times between 5°C and 70°C, and then stored for at least 12 h before measurement. For the elucidation of the protein secondary structure in the absence and presence of endotoxins, 2 mM rHSA was prepared in buffer made either from H₂O or D₂O incubated at 37°C for 0.5 h, and lipid dispersions prepared as described above were added in appropriate amounts, and further incubated at 37°C for 15 min. Afterwards, 10 μ l of these dispersions were spread on a CaF₂ infrared window, and the excess buffer was evaporated slowly at 37°C.

FTIR spectroscopy

The infrared spectroscopic measurements were performed on a 5-DX FTIR spectrometer (Nicolet Instruments, Madison, WI, USA) and on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). The lipid samples were placed in a CaF₂ cuvette with a 12.5 μ m teflon spacer. Temperature-scans were performed automatically between 10–70°C with a heating-rate of 0.6°C/min. Every 3°C, 50 interferograms were accumulated, apodized, Fourier transformed, and converted to absorbance spectra.

For strong absorption bands, the band parameters (peak position, band width, and intensity) were evaluated from the original spectra, if necessary after subtraction of the strong water bands. The position of the peak maxima was determined with a precision of better than 0.1 cm⁻¹. In the case of weak absorption bands, resolution enhancement

techniques like Fourier self-deconvolution¹² were applied after base-line subtraction. The choice of the three key parameters was done as described recently.¹³

In the case of overlapping bands, in particular for the analysis of amide I-vibration, curve fitting was applied using a modified version of the CURFIT program obtained by D. Moffat, NRC, Ottawa, Canada. An estimate of the number of band components was obtained from deconvolution of the spectra, and the curve was fitted to the original spectra after subtraction of base-lines resulting from neighbouring bands. As with the deconvolution technique, the bandshapes of the single components are superpositions of Gaussian and Lorentzian. Best fits were obtained by assuming a Gauss fraction of 0.55–0.60.

The order parameter *S* was estimated as described earlier,¹⁴ where *S* is calculated from the peak position of the symmetric stretching vibrational band ν_s of ν_s (CH_2) by:

$$S = -941.8 + 0.7217\nu_s - 7.823 \cdot 10^{-5}\nu_s^2 - 2.068 \cdot 10^{-8}\nu_s^3 \quad \text{Eq. 1}$$

Note that *S* defined in this way should be similar, but not identical, to the order parameter used in NMR spectroscopy (*S* = 1 for perfectly aligned and 0 for isotropic acyl chains).

Attenuated total reflectance (ATR)

The lipids were prepared as oriented thin multilayers as described¹⁵ by spreading a 1 mM lipid suspension – which was temperature-cycled between 5°C and 70°C several times prior to spreading – in HEPES buffer on a ZnSe ATR plate and evaporating the excess water by slow periodic movement under a nitrogen stream at room temperature. The lipid sample was placed in a closed cuvette, and the air above the sample was saturated with water vapour to maintain full hydration. Infrared ATR spectra were recorded with a mercury-cadmium-telluride (MCT) detector with a scan number of 1000 at a resolution of 2 cm^{-1} . The infrared radiation was polarized with a ZnSe wire-grid polarizer (Specac, Orpington, UK). The measurements were performed at 26°C, the intrinsic instrument temperature, in some cases also at 37°C. In the present investigation, dichroic ratios *R* for particular vibrational bands from the diglucosamine backbone at 1170, 1085, and 1045 cm^{-1} and from the antisymmetric stretch of the negatively charged phosphate $\nu_{\text{as}}(\text{PO}_2^-)$ were evaluated. The angle between the diglucosamine diphosphorylated backbone and the direction of the hydrocarbon chains can be calculated from the measured *R*, if an approximate value of *S* is known.¹⁵ As a first approximation, the *S*-value calculated from Equation 1 based on the peak position of the symmetric stretching vibration $\nu_s(\text{CH}_2)$ can be taken.

Since the hydrocarbon chains of the lipid multilayer are distributed homogeneously around the normal to the ATR plate (partial axial distribution), a tilt of the backbone with respect to the direction of the acyl chains leads to a broader orientational distribution of the backbone, which would correspond to a lower *S*-value. This implies that the acyl chains are more isotropically distributed and the estimate of *S* is an upper limit.

The water content of the samples on the ATR crystal, which is important for an exact calculation of the *z*-component of the electric field, E_z , was estimated from the ratio between the peak intensities of the main OH-stretch around 3400 cm^{-1} and the antisymmetric stretch of the methylene groups around 2920 cm^{-1} . As this ratio depends on the chemical composition of the particular lipid/protein sample at a given water content, each sample was calibrated by recording infrared spectra in transmission at various water contents and determining the corresponding peak ratios.

X-ray diffraction

X-ray diffraction measurements were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation facility HASYLAB using the double-focusing monochromator-mirror camera X33.¹⁶ Diffraction patterns in the range of the scattering vector $0.07 < s < 1 \text{ nm}^{-1}$ ($s = 2 \sin \theta / \lambda$, 2θ scattering angle and λ the wavelength = 0.15 nm) were recorded at 40°C with exposure times of 2 or 3 min using a linear detector with delay line readout.¹⁷ The *s*-axis was calibrated with tripalmitate which has a periodicity of 4.06 nm at room temperature. Details of the data acquisition and evaluation system can be found elsewhere.¹⁸ The diffraction patterns were evaluated as described previously¹⁹ assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures. The lamellar and cubic structures are most relevant here. They are characterized by the following features:

Lamellar

The reflections are grouped in equidistant ratios, *i.e.* 1, 1/2, 1/3, 1/4, *etc.* of the lamellar repeat distance d_l .

Cubic

The different space groups of these non-lamellar three-dimensional structures differ in the ratio of their spacings. The relation between reciprocal spacing $s_{\text{hkl}} = 1/d_{\text{hkl}}$ and lattice constant *a* is:

$$s_{\text{hkl}} = [(h^2 + k^2 + l^2)/a]^2 \quad \text{Eq. 2}$$

where *hkl* = Miller indices of the corresponding set of plane.

Differential scanning calorimetry (DSC)

A stock solution of 1 mg/ml of LPS dispersed in buffer at pH 6.8 was prepared. The LPS liposomes were obtained by sonication of the lipid dispersion for 10 min at 40°C. After cooling to room temperature, a certain amount of protein was added to 1 ml of lipid dispersion and the sample was gently shaken until the protein was completely dissolved. Differential scanning calorimetry (DSC) measurements were performed with a MicroCal VP scanning calorimeter (MicroCal, Inc., Northampton, MA, USA). The heating and cooling rate was 1°C/min. Heating and cooling curves were measured in the temperature interval 10–120°C. Three consecutive heating and cooling scans were measured.

Isothermal titration calorimetry (ITC)

Microcalorimetric experiments of rHSA-binding to endotoxins were performed on a MCS isothermal titration calorimeter (Microcal Inc.) as described.²⁰ The endotoxin samples prepared as described above at a concentration of 5×10^{-5} M were filled into the microcalorimetric cell and rHSA at a concentration of 5×10^{-4} M was loaded into the syringe compartment, both after thorough degassing of the suspensions. After temperature equilibration, the rHSA was titrated in 5 µl portions every 10 min into the endotoxin-containing cell, and the heat for each injection measured by the ITC instrument was plotted versus time. A control measurement in which the protein was titrated into the pure buffer indicated that the heat of dilution was negligible. The total heat signal from each experiment was subsequently determined by integrating the individual peaks and plotted against the [rHSA]:[endotoxin] molar ratio.

Zeta potential

Zeta potentials were determined with a Zeta-Sizer 4 (Malvern Instr., Herrsching, Germany) at a scattering angle of 90° from the electrophoretic mobility by laser-Doppler anemometry as described earlier.²¹ The Zeta potential was calculated according to the Helmholtz-Smoluchowski equation from the mobility of the aggregates in a driving electric field of 19.2 Vcm⁻¹. It was determined for the endotoxins (0.5 mM) at different rHSA concentrations.

Fluorescence resonance energy transfer (FRET)

The FRET assay was performed as described earlier.^{22,23} Briefly, phospholipid liposomes from phosphatidylserine

(PS) were doubly labeled with the fluorescent dyes N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine (NBD-PE) and N-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) (Molecular Probes, Eugene, OR, USA). Intercalation of unlabeled molecules into the doubly labeled liposomes leads to probe dilution and with that to a lower FRET efficiency: the emission intensity of the donor increases and that of the acceptor decreases (for clarity, only the quotient of the donor and acceptor emission intensity is shown here).

In all experiments, doubly labeled PS liposomes were prepared and after 50, 100, and 150 s recombinant LBP, LPS, and rHSA were added in different order, each at a final concentration of 0.01 mM, and the NBD donor fluorescence intensity at 531 nm was monitored for at least 300 s.

Determination of endotoxin activity by the chromogenic Limulus test

Endotoxin activity of [LPS]:[rHSA] mixtures at concentrations between 10 µg/ml and 10 pg/ml was determined by a quantitative kinetic assay based on the reactivity of Gram-negative endotoxin with *Limulus* amoebocyte lysate (LAL),²⁴ using test kits from BioWhittaker (# 50-650U). The standard endotoxin used in this test was from *Escherichia coli* (O55:B5), and 10 EU/ml corresponds to 1 ng/ml. In this assay, saturation occurs at 50 endotoxin units EU/ml, and the resolution limit is ≤ 0.2 EU/ml (maximum value for ultrapure water from embryo-transfer, Sigma).

Stimulation of human mononuclear cells (MNCs) by LPS

For an examination of the cytokine-inducing capacity of the [endotoxin]:[rHSA] mixtures, MNCs were stimulated with the latter, and TNF-α production by the cells was determined in the supernatant.

MNCs were isolated from heparinized (20 IE/ml) blood taken from healthy donors and processed directly by mixing with an equal volume of Hank's balanced solution and centrifugation on a Ficoll density gradient for 40 min (21°C, 500 g). The interphase layer of mononuclear cells was collected and washed twice in Hank's medium and once in serum-free RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were resuspended in serum-free medium, and the cell number was equilibrated at 5×10^6 N/ml. For stimulation, 200 µl/well MNCs (5×10^6 cells/ml) were transferred into 96-well culture plates. The stimuli were serially diluted in serum-free RPMI 1640 and added to the cultures at 20 µl per well. The cultures were incubated for 4 h at 37°C under 5% CO₂. Supernatants were collected after centrifugation of the

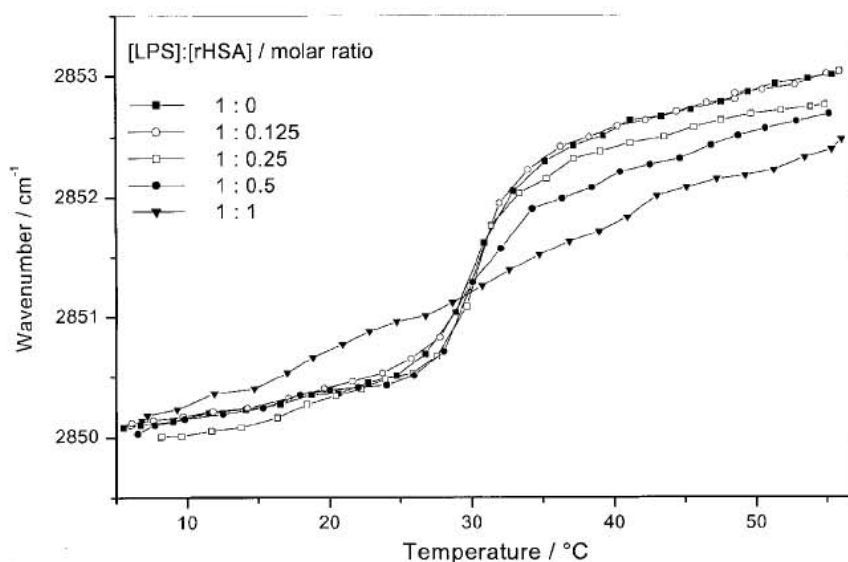


Fig. 1. Peak position of the symmetric stretching vibration of the methylene groups $\nu_s(\text{CH}_2)$ versus temperature for a 10 mM LPS Re preparation at different rHSA concentrations. In the gel (β) phase of the LPS Re acyl chains, the peak position lies at 2850 cm^{-1} , in the liquid crystalline (α) phase at 2852.5 cm^{-1} .

culture plates for 10 min at 400 g and stored at -20°C until determination of cytokine content.

Immunological determination of TNF- α in the cell supernatant was performed in a sandwich-ELISA as described elsewhere.²⁵ The 96-well plates (Greiner, Solingen, Germany) were coated with a monoclonal antibody against TNF (clone 6b from Intex AG, Switzerland). Cell culture supernatants and the standard (recombinant TNF, Intex) were diluted with buffer. After exposure to appropriately diluted test samples and serial dilutions of standard rTNF, the plates were exposed to rabbit anti-(human TNF- α) Ig peroxidase conjugate. The plates were shaken for 16–24 h at 4°C and washed 6 times in distilled water to remove the antibodies. Subsequently, the color reaction was started by addition of tetramethylbenzidine/ H_2O_2 in alcoholic solution and stopped after 5–15 min by addition of 1 N sulfuric acid. In the color reaction, the substrate is cleaved enzymatically, and the product was measured photometrically on an ELISA reader (Rainbow, Tecan, Crailsham, Germany) at a wavelength of 450 nm and the values were related to the standard. TNF- α was determined in duplicate at two different dilutions and the values were averaged.

RESULTS

$\beta \leftrightarrow \alpha$ acyl chain melting behavior

The influence of different rHSA concentrations on the gel-to-liquid crystalline ($\beta \leftrightarrow \alpha$) phase transition of the acyl chains of LPS Re and lipid A was investigated by

FTIR by evaluating the peak position of the symmetric stretching vibration of the methylene groups $\nu_s(\text{CH}_2)$ lying around 2850 cm^{-1} in the gel phase and 2852.5 cm^{-1} in the liquid crystalline phase. The results plotted for LPS Re at different molar ratios of rHSA (Fig. 1) indicated that the phase transition temperature is independent of the rHSA/LPS molar ratio. In contrast, the acyl chain fluidity decreases (rigidification) in the liquid crystalline, but increases (fluidization) in the gel phase at the highest concentrations as indicated by the decrease or the increase, respectively, of the wave number values. At 37°C , rigidification clearly takes place, this effect is of the order of 0.8 cm^{-1} at [LPS]:[rHSA] 1:1 molar.

The results obtained for the system lipid A and rHSA at different molar ratios indicated a slight increase (3°C , at lipid A:rHSA 1:0.1 molar ratio) in phase transition temperature, and a slight rigidification of the acyl chains in the liquid crystalline phase, whereas in the gel phase no change due to rHSA binding was observed (data not shown).

The phase transition behavior was also monitored with DSC. The results obtained for pure rHSA show a strong endotherm indicating denaturation in the range of $60\text{--}80^\circ\text{C}$ ($T_{\text{max}} = 68^\circ\text{C}$), and the subsequent decrease of C_p (at 85°C) indicates aggregation. The process is irreversible because in the subsequent cooling scan no peak is visible (Fig. 2A). LPS in pure form exhibits a broad endothermic reaction (around 30°C) in the heating scan and an exothermic reaction in the subsequent cooling scan with nearly no hysteretic behavior (Fig. 2B). The phase behavior was monitored also in the presence of different amounts of HSA (w:w): 70:1 molar, 30:1

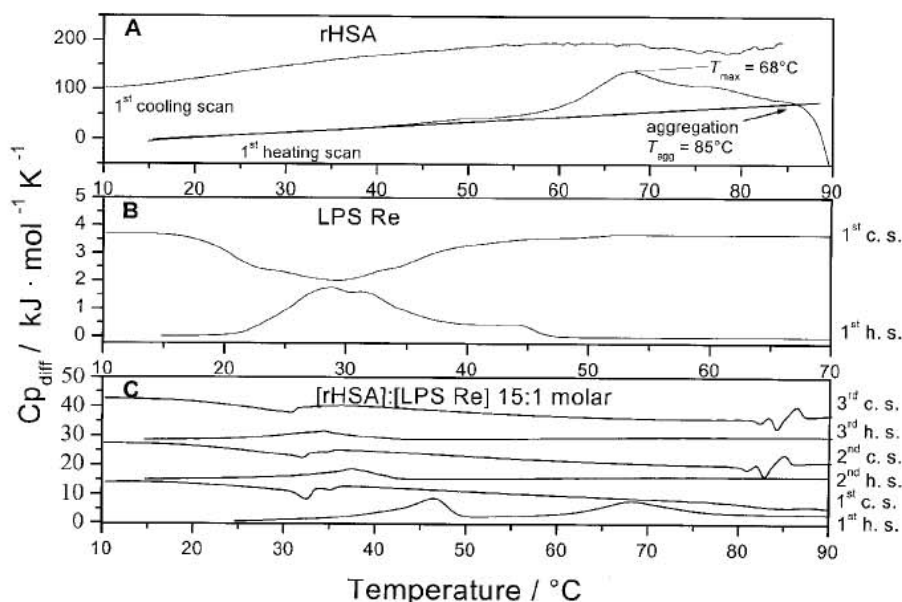


Fig. 2. DSC measurements of pure rHSA (A), LPS Re (B), and mixtures of [rHSA]:[LPS Re] 15:1 molar (C). Heating and cooling curves were measured in the temperature intervals of 10–100°C. One heating and cooling scan (A,B), and three (C) consecutive heating and cooling scans are presented (h.s. heating-scan, c.s. cooling-scan).

molar, 15:1 molar (Fig. 2C), and 4:1 molar. Clearly, due to rHSA binding the endothermic peak of pure LPS Re (around 30°C) is shifted to higher temperatures. The first heating scan is characterized by two endotherm peaks with their maxima at 46–47°C and 67–69°C. This is independent of the amount of added protein. The succeeding cooling scan revealed only one exotherm peak at 32°C. With increasing amount of HSA the maximum of the heat capacity curve of the first cooling thermogram is slightly shifted to higher temperature and the heat capacity curve is marked by two maxima. At a 4:1 molar ratio, these two maxima are found at 34°C and 37.3°C. The second heating scan shows the appearance of only one more or less structured heat capacity curve and with increasing protein content the maximum is shifted from 32°C to 40°C.

Further heating and cooling scans reveal mainly the characteristics as observed to the first cooling and second heating scan.

The peak observed for the first heating scan in the lipid-protein samples around 67–69°C is induced by protein defolding (denaturation). This can be easily concluded by comparing with the heat capacity curve of pure HSA and by the fact that with increasing amount of HSA in the sample the enthalpic contribution of the second peak increases with increasing amount of protein in the sample. Compared to pure LPS the maximum of the first peak is clearly shifted to higher temperature ($\Delta T \sim 18^\circ\text{C}$). This result indicates that the protein interacts strongly with LPS. After heating the sample above 85°C,

which is the temperature for HSA protein denaturation, binding of denatured HSA to LPS is measured in the second heating scan. This binding is not so strong. As a consequence, the perturbation of the LPS membrane by denatured protein induces a smaller temperature shift to higher temperatures.

Competition experiments with the polypeptide polymyxin B (PMB), which strongly binds to the lipid A phosphates, were performed with FTIR to examine whether the phosphates are possible binding-sites for rHSA (Fig. 3a). The results for the LPS Re, HSA, and PMB system show that PMB alone causes a strong fluidization. Addition of HSA to a mixture of LPS and PMB leads to an overall decrease of the fluidity, addition of PMB to a mixture of LPS and rHSA leads to a similar phase behavior lying between the curves of the pure agents. PMB and rHSA thus seem to have different binding sites, *i.e.* rHSA does not primarily bind to the lipid A phosphates. These results are supported by the data for the dephospho-LPS Re-rHSA system (Fig. 3b). If the phosphates were the primary binding site, no change of the phase transition behavior of dephospho LPS would be expected. Addition of HSA causes a slight increase in T_c and a rigidification, particularly in the liquid crystalline phase.

LPS backbone orientation

Infrared ATR experiments with polarized light were performed with hydrated lipid A multilayers in the absence

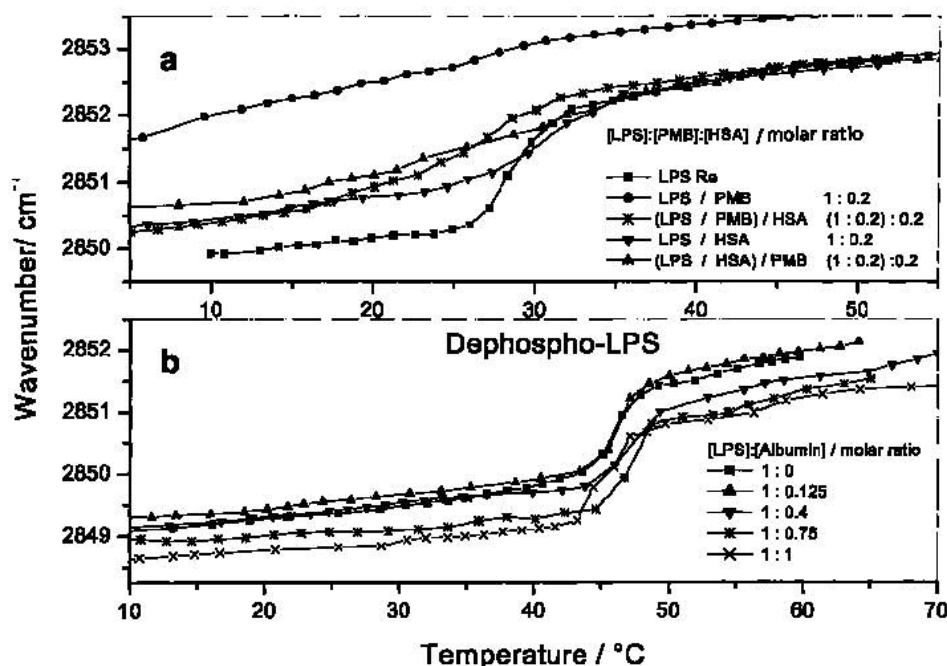


Fig. 3. Peak position of the symmetric stretching vibration of the methylene groups $\nu_s(\text{CH}_2)$ versus temperature in competition experiments with 10 mM preparations of LPS Re, PMB and rHSA (a), and for dephospho-LPS Re preparations at different rHSA concentrations (b).

and presence of rHSA to determine the influence of rHSA on particular vibrational bands of the lipid A backbone and on the tilt angle of the sugar backbone with respect to the membrane surface. The dichroic ratios R of the glucosamine vibration bands at 1060 cm⁻¹ and 1000 cm⁻¹ were calculated from measurements at 0° and 90° polarization for different [lipid A]:[rHSA] ratios. From these data and approximations of the order parameter S , a value of the angle of inclination θ of the backbone with respect to the membrane surface between 40° and 45° can be deduced, independently of the amount of rHSA (Table 1).

Table 1. Summary of infrared ATR experiments

[LPS]:[rHSA] molar ratio	Inclination angle ± SD	Dichroic ratio R ($\Delta R = 0.02$)
1:0	40.8 ± 0.9	1.0667
1:0.0625	41.5 ± 2	1.099
1:0.125	42.2 ± 0.7	1.122
1:0.25	43.8 ± 0.7	1.108
1:0.4	40.9 ± 0.7	1.143

Binding enthalpy and stoichiometry

The enthalpy of protein binding to LPS Re and their binding stoichiometry, *i.e.* the ratio at which binding saturates, was investigated by ITC (Fig. 4). At low [rHSA]:[LPS] ratios, a strong exothermic process takes place, which converges to zero in the molar range [rHSA]:[LPS] = 0.06–0.08. Saturation occurs approximately at [rHSA]:[LPS] 1:10 molar.

Lipid A aggregate structures

The conformation (shape) of the lipid A part of LPS has been shown to be a determinant of bioactivity^{26,27} and can be estimated by the type of aggregate structure adopted under near physiological conditions. In Figure 5, small-angle X-ray diffraction patterns are plotted for pure rHSA (Fig. 5a), pure lipid A (Fig. 5b), and for [lipid A]:[rHSA]

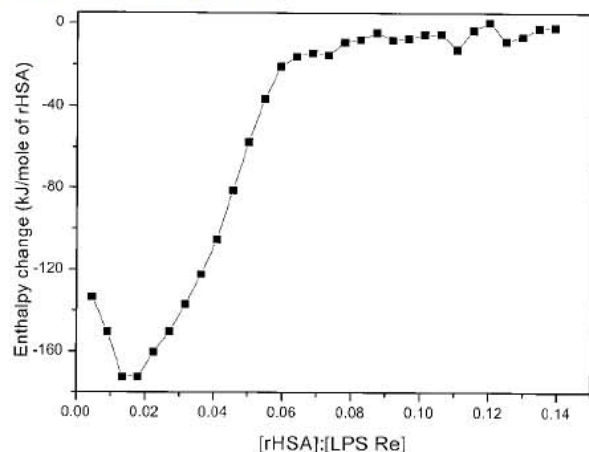


Fig. 4. Isothermal calorimetric titration of rHSA with LPS Re. The endotoxin was prepared at a concentration of 0.05 mM in the calorimetric cell, and the rHSA at a concentration of 0.5 mM in the syringe used for titration. Binding saturation is reached when the curves run horizontally.

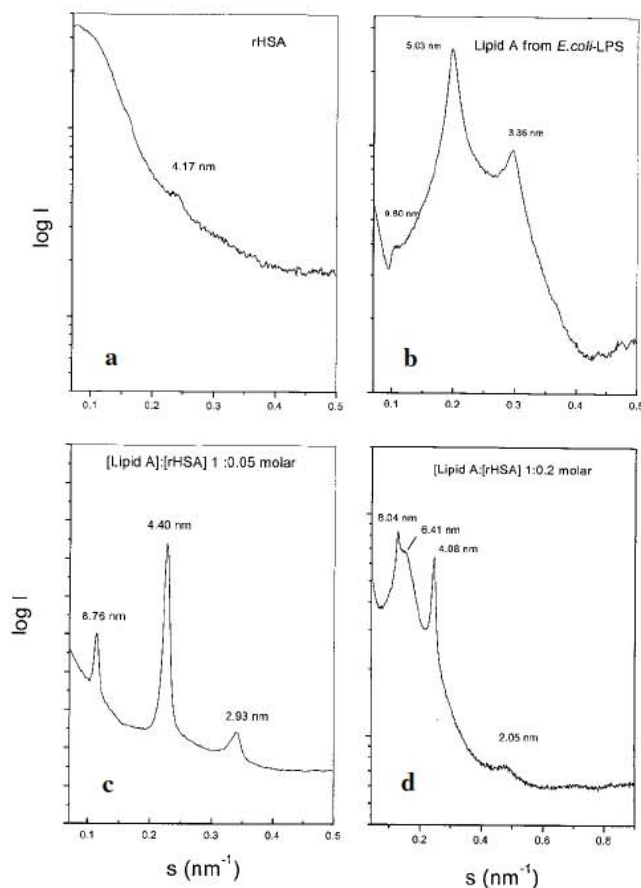


Fig. 5. Synchrotron radiation X-ray diffraction patterns of lipid A, rHSA and for [lipid A]:[rHSA] molar ratios of 1:0.05 and 1:0.2 at 90% water content. The diffraction pattern of lipid A is typical for a cubic inverted structure (b), for pure rHSA the maximum at 4.17 nm indicates aggregated albumin (a). At the lower albumin concentration (c) the aggregate structure is converted into a type of multilamellar pattern, and at the highest albumin content (d) of a non-lamellar structure.

molar ratios of 20:1 and 5:1, each at 90% water content and 37°C. The diffraction pattern of lipid A is – by analogy to previous results²⁸ – typical for a cubic inverted structure. In the presence of rHSA, the aggregate structure is converted into a different type which seems to have multilamellar character at the lower albumin concentration (Fig. 5c), but with a quite different intensity pattern where the second rather than the first order has the highest intensity. At the highest albumin content (Fig. 5d), the diffraction pattern is indicative of a non-lamellar structure which cannot be more precisely identified.

rHSA secondary structure

The secondary structure of rHSA was determined by IR-spectroscopy by analyzing the amide I-vibration (predominantly C–H stretching vibration) in the spectral

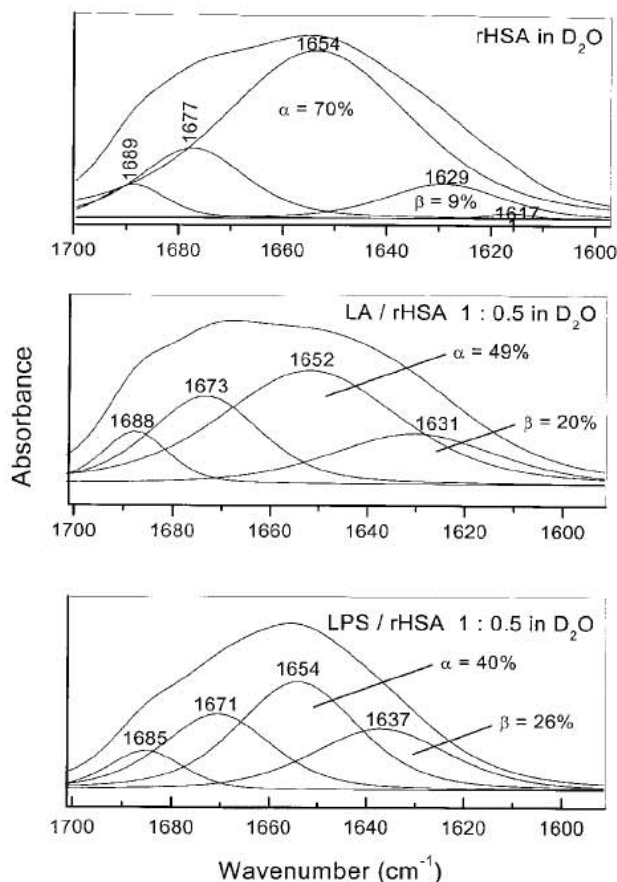


Fig. 6. Infrared spectra in the range of the amide I (predominantly C=O stretch)-vibration for hydrated samples in D₂O of rHSA and in the presence of lipid A or LPS Re ([endotoxin]:[rHSA] = 1:0.5 molar). The band component of 1652–1654 cm⁻¹ is typical for an α -helix, which retains after addition of the endotoxins, and band components resulting from β -sheets (at 1625–1640 cm⁻¹) and from β -turns and/or antiparallel β -sheets (at 1670–1680 cm⁻¹) become stronger.

range 1700–1600 cm⁻¹ in H₂O-containing as well as D₂O-containing buffer. The results for pure rHSA and in the presence of lipid A and Re-LPS ([endotoxin]:[rHSA] = 1:0.5 molar) in Figure 6 indicate a dominant α -helical structure for pure rHSA (band at 1654 cm⁻¹) which remains after addition of the endotoxins; however, band components resulting from β -sheets (at 1625–1640 cm⁻¹) and from β -turns and/or antiparallel β -sheets (at 1670–1680 cm⁻¹) become stronger.

Zeta potential

The Zeta potential as a measure for the accessible surface charges was measured by determining the electrophoretic mobility for LPS Re and lipid A in the presence of increasing amounts of rHSA. From Figure 7 it can be deduced that the pure endotoxins have a high negative

surface charge corresponding to potentials of -45 to -50 mV for LPS Re, which is only reduced by 10 mV after addition of larger amounts of rHSA. Lipid A has a higher negative surface charge corresponding to a potential of -70 mV, which is reduced by 30 mV upon addition of rHSA leading to a Zeta potential of -40 mV similar to that of LPS Re. For both compounds, the saturation of charge compensation occurs around $[\text{rHSA}]:[\text{endotoxin}] = 1:10$ molar.

Intercalation into phospholipid liposomes

It has been shown that LBP mediates the transport of LPS into negatively charged liposomes²³ which seems to be an important step in cell activation. Here, the LBP-mediated transport of LPS into phosphatidylserine (PS) as an example of a negatively charged phospholipid was determined by fluorescence resonance energy transfer (FRET) spectroscopy in the absence and presence of rHSA (Fig. 8). The three different compounds were added at $t = 50, 100$, and 150 s. Addition of LPS to PS liposomes first (Fig. 8a) followed by addition of LBP leads to an increase in NBD fluorescence intensity only after the addition of LBP and corresponds to intercalation of LBP and LPS into the target cell membrane. Addition of rHSA between that of LPS and LBP (data not shown) leads to the same result and addition of LPS between that of rHSA and LBP (Fig. 8b) also demonstrates only LBP-mediated intercalation of LPS into the

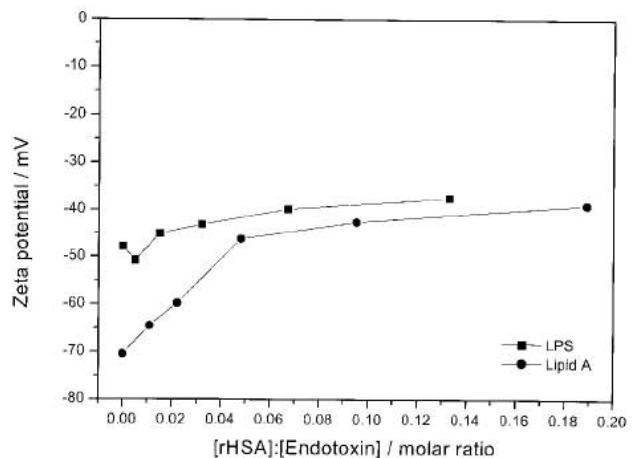


Fig. 7. Zeta potential of 0.5 mM lipid A and LPS Re preparations in dependence on different $[\text{rHSA}]:[\text{endotoxin}]$ molar ratios from the determination of the electrophoretic mobility by Laser Doppler anemometry. The data indicate only a slight compensation of the accessible surface charges of the endotoxins.

target cell membrane, but no influence of rHSA. Addition of LPS and subsequent addition of pre-incubated rHSA and LBP (Fig. 8d), and addition of pre-incubated LPS and rHSA prior to that of LBP (Fig. 8c) gives the same result as in the absence of rHSA (Fig. 8a). It can thus be concluded that rHSA does not have any influence on the LBP-mediated intercalation of LBP and LPS into the PS liposomes.

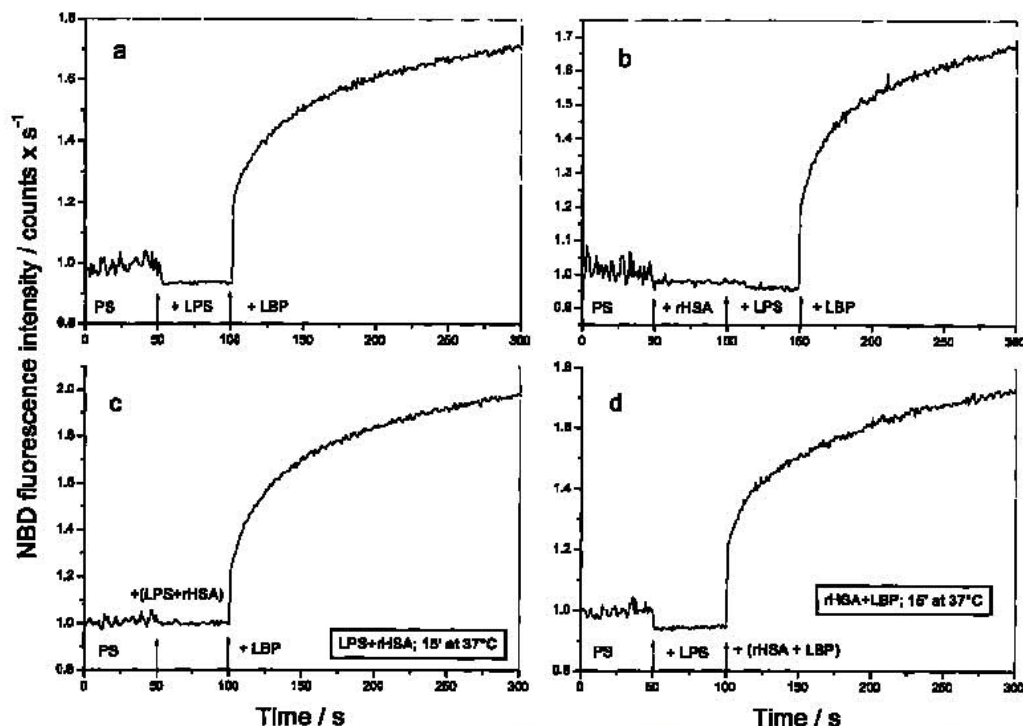


Fig. 8. Quotient of the fluorescence intensity at 531 nm of doubly labeled liposomes from PS versus time after incubation with LPS Re and subsequent addition of LBP (a), after incubation with rHSA and subsequent addition of LPS and LBP (b), after incubation with a pre-incubated mixture of LPS and rHSA and subsequent addition of LBP (c), after incubation with LPS and subsequent addition of a pre-incubated mixture of rHSA and LBP (d).

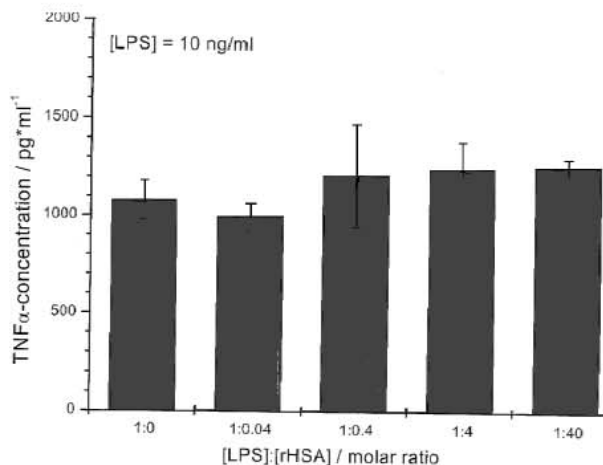


Fig. 9. LPS- induced TNF- α production in human mononuclear cells by 10 ng/ml LPS Re and at different [LPS]:[rHSA] molar ratios. The error bars (standard deviation) results from the determination of TNF- α in duplicate at two different dilutions. The data are representative of three independent measurements.

Biological activity

LPS-induced TNF- α production in mononuclear cells was investigated at different rHSA concentrations at [LPS] = 10 ng/ml. As shown in Figure 9 for one typical experiment, only a slight increase in TNF- α production is observed at excess rHSA, [LPS]:[rHSA] 1:4 and 1:40. This was confirmed in two further measurements. In contrast, the activity in the *Limulus* assay (Fig. 10) indicates a decrease of LAL reactivity at lower relative concentrations of rHSA, but again an increase at higher

amounts, which in the case of 100 pg/ml LPS reaches the original activity of pure LPS.

DISCUSSION

The binding of rHSA to the model endotoxins lipid A and Re-LPS was characterized by a variety of physical techniques and was correlated with biological effects. The data clearly indicate a predominantly non-electrostatic binding, deduced from competition measurements with PMB (Fig. 3a), the phase transition measurements with dephospho LPS (Fig. 3b), and the Zeta potential data (Fig. 7). However, a direct hydrophobic interaction is improbable since the FRET spectroscopic data (Fig. 8) do not indicate any preference for rHSA to incorporate into target phospholipid membranes by itself nor indicate any influence – when incubated with LPS – to change the LBP-induced intercalation of LPS into the target cell membranes. These results seem to contrast with those of the phase transition measurements of Re-LPS (Fig. 1), which indicate a fluidizing effect in the gel and a rigidifying effect in the liquid crystalline phase, respectively, and a strong broadening and shift to higher temperatures (Fig. 2). This behavior is similar to that of cholesterol in phospholipid membranes,²⁹ and this analogy may be the key to understanding rHSA binding to LPS since it may be deduced that the albumin molecule aligns parallel to the long axis of the LPS molecule from the backbone down into the hydrophobic moiety.

Saturation of binding occurs around [rHSA]:[LPS] 1:10 molar (Figs 4 & 7), i.e. a high number of LPS molecules can be bound by each albumin molecule. This is much

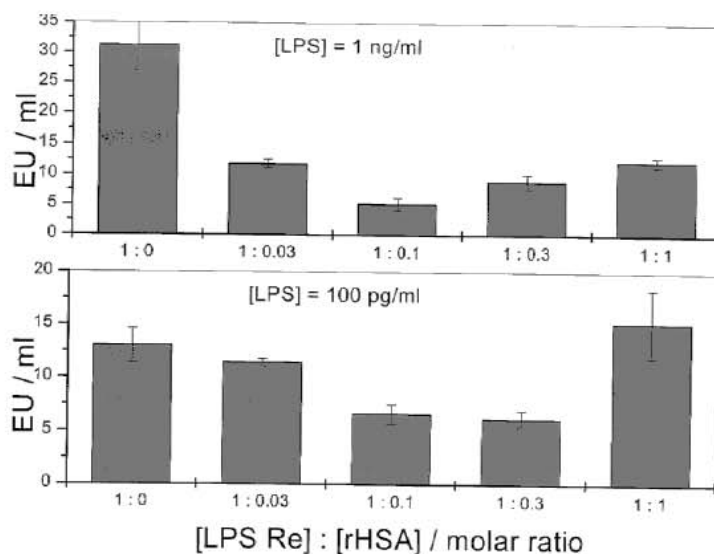


Fig. 10. Endotoxin activity in the chromogenic *Limulus* amoebocyte lysate assay at two LPS Re concentrations (1 ng/ml and 100 pg/ml) and different concentrations of rHSA.

higher than for other LPS-binding proteins. It has been reported that lactoferrin, lysozyme, and hemoglobin each have a binding stoichiometry of [protein]:[LPS] around 1:3 molar.^{21,30,31} The first two proteins lead to a significant decrease of the LPS immunostimulating activity, while the latter increases it. The amounts of endotoxin molecules bound to the different proteins are thus clearly unrelated to their ability to interact with target cells. This is confirmed also by the results from the secondary structures of the proteins. Recombinant albumin has a predominantly α -helical structure (70%, in excellent agreement with the data in the Protein Data Bank, structure 1A06). Binding of albumin to the endotoxins leads to a decrease of the α -helical content in favour of β -sheet structures (Fig. 6). Comparison with other proteins indicates that the α -helical structure of lysozyme is converted into an unordered structure in the presence of LPS, while the β -substructure remains unchanged.³⁰ For hemoglobin, the dominant α -helical structure remains unchanged on LPS binding.³¹ Finally, for lactoferrin, α -helices (38%) as well as β -sheets (25%) are found which are not altered upon LPS binding.²¹ These last two examples indicate that, although the secondary structure of the protein is not changed, in one case the LPS-stimulatory activity is enhanced and in the other case weakened.

The aggregate structure of lipid A – cubic inverted (Fig. 5a) as already observed previously²⁸ – changes into a non-lamellar structure exhibiting some lamellar characteristics, in particular at lower albumin content (Fig. 5c). An unequivocal assignment to a defined aggregate structure is, however, not possible, as it is neither a lamellar nor a cubic phase. Concomitantly, the head-group orientation of the diglucosamine backbone of lipid A, which was shown earlier to have an inclination angle of 40° and higher with respect to the membrane surface for biologically active lipid A,¹⁵ does not change upon albumin binding. These observations should be discussed in the context of the previously published conformational concept.²⁷ It was found that endotoxically active LPS, such as those from enterobacterial source with a hexa- to hepta-acylated lipid A, adopt a cubic inverted structure from which a conical shape of the lipid A moiety – higher cross-section of the hydrophobic as compared to the hydrophilic moiety – can be deduced. Concomitantly, the diglucosamine backbone adopts a high (> 40°) inclination angle with respect to the membrane surface.¹⁵ Endotoxically inactive LPSs, such as that from *Rhodobacter capsulatus* or those with a tetra- or penta-acylated lipid A, have a lamellar aggregate structure, corresponding to a cylindrical shape of the lipid A moiety with a small angle (< 20°) between the backbone and the membrane surface. The data for lactoferrin and lysozyme and the polypeptide polymyxin B are in agreement with this concept.^{20,21,30} The binding of these drugs to LPS leads to a conversion of the cubic

inverted structure of the lipid A part into a multilamellar structure, *i.e.* a conversion from a conical into a cylindrical shape. Also, for lactoferrin the inclination angle is reduced (for lysozyme and PMB no such measurements were performed). Thus, lipid A is converted from an active into an inactive conformation. In contrast, for the binding of hemoglobin to lipid A, the cubic structure of the latter is converted into another cubic structure and the inclination angle of the backbone is not reduced but even enhanced.³¹ Concomitantly, the immunostimulatory activity of LPS shows an increase. In this case, the active conformation becomes even more active.

These interpretations are in full accordance for rHSA: there is no transition of the aggregate structure into a multilamellar nor into another cubic structure, and the inclination angle does not change significantly. In parallel, the cytokine-inducing activity shows only a slight increase at the highest rHSA-concentrations (Fig. 9).

The results for the *Limulus* assay do not exhibit a consistent behavior for the two LPS concentrations (Fig. 10), and in particular do not parallel the cytokine production measurements. These data are in accordance with those found for the binding of LPS to lactoferrin and to hemoglobin; in both cases, a LPS concentration-dependent change of the LAL activity was observed leading to a decrease or to an increase, respectively, of the LAL reaction. It must be emphasized that the *Limulus* test shows also high activity for lipid A preparations such as tetra-acyl and penta-acyl lipid A, which are completely inactive in cytokine assays (unpublished results).

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