Cross-linked Hemoglobin Converts Endotoxically Inactive Pentaacyl Endotoxins into a Physiologically Active Conformation*

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The interaction of purified αα cross-linked hemoglobin (ααHb) with a pentaacylated mutant lipopolysaccharide (pLPS) and the corresponding lipid A (pLA) was studied biophysically and the effects correlated with data from biological assays, i.e. cytokine induction (tumor necrosis factor-α) in human mononuclear cells and the Limulus amebocyte lysate assay. Fourier transform infrared spectroscopic and Zeta-Sizer experiments indicated an electrostatic as well as a non-electrostatic binding of ααHb to the hydrophilic and to the hydrophobic moieties of the endotoxins with an increase of the inclination angle of the pLA backbone, with respect to the membrane surface, from 25° to more than 50°. Small angle synchrotron radiation x-ray diffraction measurements indicated a reorientation of the lipid A aggregates from a multimamellar into a cubic structure as a result of ααHb interaction. Thus, in the absence of ααHb, the molecular shape of the pentaacyl samples was cylindrical with a moderate inclination of the di-glucosamine backbone, whereas, in the presence of the protein, the shape was conical, and the inclination angle was high. The cytokine-inducing capability in human mononuclear cells, negligible for the pure pentaacylated compounds, increased markedly in the presence of ααHb in a concentration-dependent manner. In the Limulus assay, the pentaacylated samples were active a priori, and their activity was enhanced following binding to ααHb, at least at the highest protein concentrations. The data can be understood in the light of a reaggregation of the endotoxins because of ααHb binding, with the endotoxin backbones then readily accessible for serum and membrane proteins. By using fluorescence resonance energy transfer spectroscopy, an uptake of the endotoxin-Hb complex into phospholipid liposomes was observed, which provides a basis for cell activation.

Bacterial endotoxins (lipopolysaccharide, LPS),1 with their hydrophobic membrane anchor lipid A constituting the major lipid component of the outer membrane of Gram-negative bacteria, are biologically highly active in mammals, which may be beneficial at lower but pathophysiological at higher concentrations, because of excessive cytokine production by cells of the immune system (1). Endotoxins are one of the major agents that may cause septic shock; currently, no successful anti-endotoxin therapies are available. LPS consists of a sugar portion with varying lengths of oligo- or polysaccharide chains depending on the type of bacterial mutant, i.e. rough mutant LPS Re to Ra or smooth form LPS. The polysaccharide component is covalently linked to the hydrophobic anchor of LPS, lipid A (2–4). It is known that the lipid A moiety represents the “endotoxic principle” of LPS (1). There are various endotoxin-binding proteins, which may have considerable biological importance. The binding of these proteins to endotoxins is frequently only poorly characterized, but an understanding of the processes involved is important in light of the modifications of the LPS-induced responses (5–7). An activity-enhancing protein is hemoglobin (Hb), the four-subunit globular oxygen-carrying protein of vertebrates and some invertebrates. It is present in the red blood cells specialized for oxygen transport, which contain a high concentration of hemoglobin in their cytoplasm. Hb solutions are potentially clinically useful for resuscitation, elective surgery, shock, cancer therapy, sickle cell anemia, coronary balloon angioplasty, stroke, and myocardial ischemia (8, 9). In previous reports, it was found that the interaction of Hb with LPS results in a significant increase of the biological activity of LPS (8, 10–12). In addition, the lethal toxicity of LPS for rodents is enhanced in the presence of Hb (13). The enhancement of the biological response was found to be associated with a Hb-induced disaggregation of LPS (11, 14).

Recently, we characterized the binding of Hb with enterobacterial LPS and lipid A, and found that the increase of the LPS-induced cytokine induction was the result of an enhancement of the “endotoxic conformation” of the lipid A component of LPS (6). In that report, enterobacterial endotoxins were used, which a priori were highly active. From these results the question arises whether endotoxins that are only weakly active or completely inactive in biological systems because of a change in chemical structure also may become active by binding with Hb. To determine this, we selected a mutant pentaacylated LPS (pLPS) and the corresponding lipid A (pLA) from Esche-
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richia coli strain F515, which has been shown to be agonistically inactive, i.e. induced cytokine production, if at all, only slightly, but in contrast, was antagonistically active (15). In the current study, we have found that the interaction of cross-linked ααHb with the pentacycated endotoxins has an electrostatic as well as a non-electrostatic hydrophobic character, which are associated with conversion of the endotoxins into an active form.

MATERIALS AND METHODS

Lipids and Reagents—Lipopolysaccharide from a mutant of the deep rough mutant Re E. coli F515, which produced only a pentacycated lipid A, in which the 3-OH-bound myristic acid at position 3' of the non-reducing diglucosamine is lacking (16), was extracted from bacteria grown at 37 °C by the phenol/chloroform/petrol ether method (16), purified, and lyophilized. Free lipid A was isolated by acetate buffer treatment of the mutant LPS F515. After isolation, lipid A was purified and converted to the triethylamine salt form. The known chemical structures of lipid A from LPS F515 were confirmed by the analysis of the sample preparation of liposomes resembling the composition of the cell membranes of Limulus polyphemus and that the estimate of the acyl chains is known (15). As a first approximation, the angle θ represents the angle between the diglucosamine diphasphorylated backbone and the direction of the hydrocarbon chains, and can be calculated from the measured S1, if an approximate value of the order parameter S is known (15). As a first approximation, the angle θ represents the angle between the direction of the backbone, which would correspond to a lower S value. This implies that the acyl chains are more isotropically distributed and that 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 c-component of the electric field, E, was estimated from the ratio between the peak intensities of the main OH-stretch around 3400 cm⁻¹ and the antisymmetric stretch of the acyl groups around 1710 cm⁻¹. For overlapping bands, in particular for the analysis of amide I- vibrations, a number of 0.55-0.60. The order parameter S, a measure of the parallel alignment of the endotoxin molecules, was estimated as described earlier (21), where S is calculated from the peak position of the symmetric stretching vibration ω (cm⁻¹) and the skewing of the bands from the diglucosamine backbone were evaluated at 1170, 1085, and 1045 cm⁻¹. The angle θ represents the angle between the diglucosamine diphasphorylated backbone and the direction of the hydrocarbon chains, and can be calculated from the measured S1, if an approximate value of the order parameter S is known (15). As a first approximation, the angle θ represents the angle between the direction of the backbone, which would correspond to a lower S value. This implies that the acyl chains are more isotropically distributed and that the estimate of S is an upper limit.

For overlapping bands, in particular for the analysis of amide I-vibrations, the curve fitting was carried out using a modified version of the CURFIT program obtained from D. Moffat (National Research Council, Ottawa, Canada). An estimate of the number of band components was obtained from deconvolution of the spectra (20), and the curve was fitted to the original spectra after subtraction of base lines resulting from neighboring bands. As with the deconvolution technique, the band shapes 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 lipid samples were placed in a CaF2 cuvette with a 12.5-μm Teflon spacer. Temperature scans were performed automatically from 10 to 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.

FTIR Spectroscopy—The infrared spectroscopic measurements were performed on a FTIR spectrometer (5-DX, Nicolet Instruments, Madison, WI). Methanol, 2,2,2-trifluoroethanol, and perfluorooctane were used as solvents.

Fluorescence Resonance Energy Transfer (FRET)—The FRET assay was performed as described previously (5, 23). Briefly, phospholipid liposomes similar to the composition of the macrophage plasma membrane, PLαLα, were doubly labeled with the fluorescent dyes N-(7-nitrobenz-2-
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oxa-1,3-diethyl-4-(phosphatoxy)cholestanol (NBD-PE) and N-lysyl-
the phosphocholine, N-succinimide. (Molecular Probes, Eugene, OR). Interlacing of unlabeled molecules into the doubly labelled liposomes leads to probe dilution, thus inducing 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 has been shown here).

In all experiments, doubly labelled PLAM liposomes (0.01 mM) were prepared and after 50 s, LPS Re, αHb, or co-incubated LPS + αHb (0.5 h at 37 °C) was added, and the NBD-PE donor fluorescence intensity at 531 nm was then monitored for at least 300 s. The final molar ratios of PLAM, LPS, and αHb were 1:1:0.05.

X-ray Diffraction—X-ray diffraction measurements were performed at the Peacock Molecular Biology Laboratory outstation at the Hamburger synchrotron radiation facility HASYLAB, using the double focusing monochromator-mirror camera X53 (23). Diffraction patterns in the range of the scattering vector 0.017 ≤ s ≤ 1 nm−1 (s = 2 sin θ/λ, 2θ the 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 (26). The x 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 (27). The diffraction patterns were evaluated as described previously (28), assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures.

Here, multilamellar and cubic structures are of particular relevance, and can be characterized by the following features. For multilamellar structures, the reflections are grouped in equidistant ratios, i.e., 1/2, 1/3, 1/4, etc., of the lamellar repeat distance d0. For cubic structures, these are non-lamellar three-dimensional structures. Their various space groups differ in the ratio of their spacings. The relationship between reciprocal spacing s and lattice constant, a, is s = 1/λ. For cubic structures, the Miller indices of the corresponding set of planes.

Determination of Endotoxin Activity by the Chromogenic Limulus Test—Endotoxin activity of [LPS:αHb] mixtures at concentrations between 10 µg/ml and 100 µg/ml for both compounds was determined by a chromogenic Limulus assay based on the reactivity of Gram-negative endotoxin with Limulus amebocyte lysate (LAL) (18, 29) using test kits 50-650U from BioWhittaker (Walkersville, MD). LPS was pre-incubated with αHb in a concentration ratio range of 1:0 to 1:30 M. The measurements were performed in duplicate, and the presented results are the mean of the two determinations. In this assay, saturation occurs at 50 endotoxin units (EU)-ml−1, and the resolution limit is <0.2 (EU)-ml−1. The endotoxin used for determining the standard curve (sensitivity versus endotoxin concentration) was from E. coli O55:B5 (Sigma).

The red color of αHb might interfere in the chromogenic Limulus test. However, at the αHb concentrations used here, there was no measurable disturbance.

Stimulation of Human Mononuclear Cells—For an examination of the cytokine-inducing capacity of the [endotoxin:αHb] mixtures, human mononuclear cells (MNC) were stimulated with solutions of endotoxin and αHb and the TNFα production of the cells was then determined in the supernatant. MNC were isolated from heparinized (20 ml blood taken from healthy donors and processed directly by centrifugation on a Ficoll density gradient for 40 min (21 °C, 500 × g). The interphase layer of MNC was collected and washed twice in Hank’s medium and then once in serum-free RPMI 1640, which contained 2 mm l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were resuspended in serum-free medium, and the cell number was equilibrated at 5 × 106/ml. For stimulation, 200 µl/well MNC (5 × 106 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/well. The cultures were then incubated for 4 h at 37 °C under 5% CO2. Supernatants were collected after centrifugation of the culture plates for 10 min at 1000 × g and stored at −20 °C until determination of cytokine content.

Immunological determination of TNFα in the cell supernatant was performed in a sandwich enzyme-linked immunosorbent assay as described elsewhere (30). 96-well plates (Greiner, Solingen, Germany) were coated with a monoclonal antibody against TNF (clone 6B6, from Immunotech, Muttenz, Switzerland). Cell culture supernatants and the standard (recombinant TNFα; R&D Systems) were diluted with buffer. After exposure to appropriately diluted test samples or serial dilutions of standard rTNFα, the plates were exposed to rabbit anti-human TNFα Ig-peroxidase conjugate. The plates then were shaken for 16–24 h at 4 °C and subsequently washed six times in distilled water to remove the free antibodies. Subsequently, the colorimetric reaction was started by addition of tetramethylbenzidine/H2O2 in alcoholic solution and stopped after 5–15 min by addition of 1 N sulfuric acid. In the colorimetric reaction, the substrate is cleaved enzymatically, and the product was measured photometrically on an enzyme-linked immunosorbent assay reader (Rainbow, Tecan, Crailsheim, Germany) at a wavelength of 450 nm; the values were related to the standard. TNFα was determined in duplicate at two different dilutions, and the values were averaged.

RESULTS

In the current investigation, the interaction of αHb was studied with pLPS and pLA. Because lipid A is known to represent the “endotoxic” principle of LPS, most experiments were performed with PLA, and in selected cases also with pLPS from a deep rough mutant of E. coli.

β ⇔ α Acyl Chain Melting Behavior—The influence of different αLAc concentrations on the gel to liquid crystalline phase transition of the acyl chains of pLA was investigated utilizing FTIR, by evaluating the peak position of the symmetric stretching vibration of the methylene groups νCH2 lying around 2850 cm−1 in the gel phase and 2852.5 cm−1 in the liquid crystalline phase. The results plotted for pLA at different αLAc concentrations (Fig. 1A) exhibited a clear increase in the phase transition temperature Tc from 26 to 33 °C at the highest αLAc concentration. This effect was accompanied by a decrease of acyl chain fluidity (rigidification), as indicated by the decrease of the wave number values. At 37 °C, the latter decreased from 2852.5 to 2851.8 cm−1.

Competition measurements were performed with the catonic polypeptide polymyxin B (PMB), which is known to bind to the lipid A phosphates. By using 45Ca-displacement measurements at a pLA monolayer at the air-water interface, PMB has been shown to bind more strongly to pLA than αHb. As demonstrated in Fig. 1B, the two substances, αHb and PMB, each act oppositely; the former rigidifying, the latter fluidizing. Incubation of pLA initially with PMB and subsequently with αHb, as well as the opposite sequence, resulted in very similar curves lying between those of the individual samples. This indicated that the phosphates cannot be the only binding sites for αHb, because then PMB would have replaced the protein completely from these, leading to similar curves as for pLA/PMB alone.

Lipid A Backbone Orientation—Infrared ATR experiments with polarized light were performed with hydrated pLA multilayers, in the absence and presence of different concentrations of αHb, for the determination of the tilt angle of the sugar backbone with respect to the membrane surface. From the measurements for different [lipid A]:[αHb] ratios, at 0 and 90° polarization, the dichroic ratios, R, of the vibrations of the diglucosamine with the bands at 1060 and 1000 cm−1 can be calculated. From these data and approximations of the order parameter S, the inclination angle θ of the backbone with respect to the membrane surface can be deduced. The evaluation showed that, in the absence of αHb, the diglucosamine backbone of pLA has a tilt against the membrane surface in the range of 22–30°, which was increased to values in the range of 45–52° with increasing concentrations of αHb (data not shown).

Differential Scanning Calorimetry—The pLPS:αHb interaction was studied calorimetrically by measuring the specific excess heat, Cp, of the pure compounds and various mixtures versus temperature. The first heating scan of pure αHb demonstrated a broad coexistence temperature range from −30 to

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80 °C (Fig. 2A). Two maxima were observed in the heat capacity curve, the first one at ~63.5 °C and the second one at ~75 °C. At ~63.5 °C a definite part of the protein melted. However, full denaturation was not observed. A certain, but actually unknown, protein domain still remained in its native state and was denatured only at a higher temperature (~75 °C). Thus, the thermal stability of the domains differs by ~12 °C. At temperatures higher than 85 °C, full denaturation and aggregation were observed. Heating of ααHb above 85 °C induced irreversible protein denaturation as was deduced from the strong decline of the curve and from the second heating scan (after cooling down to 15 °C), which showed only a straight line over the entire temperature range (data not shown). Native Hb, in contrast, exhibited a melting point around 60 °C and subsequent denaturation starting at temperatures lower than 70 °C (Fig. 2A).

The heat capacity curve of the first heating scan of pLPS was characterized by a broad phase transition ($t_{1/2}$ = 7.2 °C) with its maximum at 25.3 °C and a shoulder at ~28.3 °C (Fig. 2B). The interaction of pLPS with ααHb (incubated at room temperature before the DSC scan) at various pLPS to protein ratios (Fig. 2B) showed that the presence of the protein induces stabilization of the lipid gel phase. At a pLPS to protein ratio of 1:0.003 M, the maximum of the lipid heat capacity curve was shifted from 25.3 to 28.2 °C. A similar temperature shift of $\Delta T$ = 3.3 °C to a higher temperature was observed by decreasing the ratio from 1:0.006 to 1:0.015 M. A further increase of the protein content to 1:0.03 (33:1) M induced only a slight temperature shift compared with the lower ααHb concentration. Thus, it can be tentatively concluded that at a ratio of 1:0.015, molar saturation of the lipid to protein interactions is obtained. In all thermograms of the first heating scan, an endothermic effect be-
between 50 and 80 °C was detected, indicating the presence of free, unbound protein (data not shown in Fig. 2B). In the second and further heating scans (after cooling down to 15 °C), the phase transition was much broader and the maximum of the heat capacity curve was shifted to lower temperatures (data not shown). In this case, an interaction of the lipid with the denatured protein and not with the native protein, as in the first heating scan, takes place. This phenomenon has been described previously (24). In contrast to the phase transition temperature, the presence of the protein had basically no influence on the gel to liquid crystalline phase transition enthalpy (see Fig. 2B); the enthalpy changes lie at 28 ± 2 kJ/mol for all mixtures.

Zeta Potential—The zeta potential is an indicator for accessible surface charges of endotoxins and was determined for pLA at different concentrations of [HA2]. From Fig. 3 it can be deduced that [HA2] was able to partially compensate for the negative charges of pLA, a conclusion based on the reduction of the zeta potential from ~70 to ~20 mV. This process was essentially completed at a molar ratio of approximately [HA2]:[pLA] = 0.2; further addition of the protein induced only very slight additional changes. Because the red color of Hb may interfere with the measuring wavelength of the helium-neon laser (~632.8 nm), as controls, the effects of latex particles (300 nm diameter) and phosphatidylcholine liposomes also were investigated in the presence of different concentrations of [HA2] (data not shown). In the case of latex particles (UZeta = -47 mV) and for phosphatidylcholine liposomes (UZeta = -10 mV), the zeta potential and hence the surface charge exhibited only a slight reduction to ~40 and ~5 mV, respectively, after addition of the maximum concentration of [HA2].

Aggregate Structure of pLA—Small angle diffraction patterns of pLA, in the absence and presence of [HA2], were recorded at 37 °C and at 90% water content, to determine the supramolecular aggregate structure of lipid A (28, 31, 32). The diffraction pattern of pure pentaacyl lipid A was indicative of a multilamellar structure, which can be concluded from the occurrence of the periodicity at d1 = 4.91 nm and the fourth order (Fig. 4a). In the presence of [HA2] ([pLA]:[ααHb] 3:1 m; Fig. 4b), the aggregate structure changed into a cubic inverted structure, which can be deduced from the occurrence of the periodicity aQ = (10.8 ± 0.6) nm and further reflections at 7.75 nm = aQ/2, 5.85 nm = aQ/3, 3.89 nm = aQ/8, and 2.94 nm = aQ/14 (Fig. 4b). Binding of [ααHb] to pLA thus leads to
a change from a highly aggregated structure with a cylindrical conformation of the single molecules, corresponding to the multilamellar structure, into a less aggregated phase with a conical shape, and with a higher cross-section of the hydrophobic than the hydrophilic moiety, corresponding to the cubic phase.

**Hb Secondary Structure**

The secondary structure of Hb was determined by IR spectroscopy, by analyzing the amide I vibration (predominantly C=O stretching vibration) in the spectral range 1700–1600 cm$^{-1}$ in D$_2$O-containing buffer. The spectra of purified Hb (Fig. 5, top panel) demonstrated a main band at 1660 cm$^{-1}$, indicating a dominant α-helical structure; those at 1645 and 1678 cm$^{-1}$ are indicative of β-sheet and β-turns, respectively. Under all conditions is indicative of the occurrence of random coils (in D$_2$O). From these data we can deduce that binding of pLA to Hb leads to an only slight change in the secondary structure from a pure α-helical structure to one with also some β-sheet structures. Similar results were obtained for the system pLPS and Hb (data not shown).

**Fluorescence Resonance Energy Transfer Spectroscopy**

FRET was applied to test the possibility whether αHb or LPS-αHb complexes may incorporate into phospholipid liposomes corresponding to the composition of human macrophages (PL$_{49:8}$). In Fig. 6, the results are shown after addition of pLPS, αHb, or [pLPS]:[αHb] mixtures to the liposomes PL$_{49:8}$ at time 50 s. Clearly, for pure pLPS a decrease of the fluorescence signal takes place as a result of dilution, whereas for pure αHb and even greater for the pLPS-αHb complex, a strong increase in fluorescence intensity was observed, which indicates an intercalation of the samples into the liposomes. The data for the pLPS-αHb complex further support the conclusion that incubation of αHb with pLPS decreased the aggregation of pLPS. Some experiments were also performed with chole-
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**DISCUSSION**

In previous publications, considerable increases of the biological activities of LPS in the LAL test (8, 11, 12, 35), production of mononuclear cell tissue factor (35), and stimulation of endothelial cell tissue factor factor production (36, 37) as a result of Hb binding to LPS were described. Furthermore, an increase of endotoxin binding to endothelial cells and increased lethal toxicity were reported (13, 36). Our current data indicate a strong interaction between αHb and both pLPS and pLA. This binding leads to a significant rigidification of the lipid A acyl chains (Fig. 1A), stabilization of the lipid gel phase (Fig 2B), a dramatic increase of the tilt angle of the diglucosamine backbone with respect to the membrane surface from 20–30° to more than 45°, a partial compensation of the surface charges of pLA (Fig. 3), a slight change of the secondary structure of αHb with a nearly unchanged α-helical structure and slightly increased amount of β-sheet structures (Fig. 5), and a change of the aggregate structure of pLA from a multilamellar into a cubic one (Fig. 4). The binding groups in the pentaacyl complexes are not only the lipid A phosphates but also varying fatty acids or other compounds.

The transitions observed in the DSC experiments indicate a very complex protein melting as compared, for example, to human serum albumin (23). The partial melting temperatures at 63.5 and 75 °C and the high denaturation temperature of 85 °C for αHb were 10 °C and 5 °C higher, respectively, as compared to native Hb. The mean and standard deviation are based on the data from the determination of TNFα in duplicate at two different dilutions. The data are representative of three independent sets of measurements; p < 0.0005 (***) versus 1.0 (the leftmost pair of columns). The mean and standard deviation are based on the data from the determination of TNFα in duplicate at two different dilutions. The data are representative of three independent sets of measurements; p < 0.0005 (***) versus 1.0 (the leftmost pair of columns).
stabilization of the protein by the cross-link. The increase of the acyl chain melting temperature and the transition broadness for pLPS as a result of αHb binding, observable already at [pLPS]:[αHb] = 1:0.003 M (Fig. 2B), emphasize the strength of the binding. However, the observation of nearly unchanged phase transition enthalpies indicates that no strong disturbance of the hydrophobic moiety of the endotoxins takes place. The observed lack of a clear change of the secondary structure of αHb (Fig. 5) is in accordance with the data of Kaca et al. (40), who did not observe a change in the α-helical content of 53% (as compared with 45–55% from Fig. 5) upon protein binding. Associated with the described changes in the physicochemical characteristics is, at least at the highest concentrations of αHb tested, a considerable increase in the cytokine production induced by αHb binding to the pentaacylated samples (Fig. 7). This was reflected similarly in increased activity in the Limulus amebocyte lysate assay (Fig. 8) at the highest level of αHb tested.

It should be noted, however, that the cytokine data show an enhancing effect starting at pLA:αHb = 1:4 M, whereas in the physical experiments the relative concentration of Hb was much lower for the expression of the corresponding effect. For an interpretation it must be recognized that the absolute concentrations in the physical systems are much higher than in the biological ones, which cannot be avoided because of the different sensitivity limits. Furthermore, the geometries of the systems are different. The physical systems are pure aggregate/liposome solutions, whereas the cellular systems are based on cells that adhere to the wells and are accessible only from one side. Additionally, it can be assumed that, apart from the incorporation of Hb into target membranes such as those of immune cells or of liposomal membranes (Fig. 6), the protein binds to the mononuclear cells without incorporation as well as to the wells in the bioassays. Thus, fewer Hb molecules are available for intercalation into the cells.

It should be emphasized that the activation of the clotting cascade in the Limulus assay does not necessarily parallel other biological test systems. We have found that, after binding of the endogenous proteins lactoferrin (LF) (5) or recombinant human serum albumin (rHSA) (23) to endotoxins, the effects of LPS on the production by human mononuclear cells of cytokines such as TNFα and interleukin-6 was reduced in the case of LPS or enhanced very slightly for rHSA, but these changes did not correlate with the activity in the Limulus test (there was a concentration-dependent decrease or increase for LF and decrease for rHSA). A correlation of cytokine induction with the LAL assay was found only for the system LPS-HDL, which could be explained by similar target structures for the proteins in the lipid A backbone, in particular the 4'-phosphate diglucosamine backbone (41). Based on these observations, from the parallelism of the biological data in Figs. 7 and 8, it can be concluded that the latter functional groups in the pLA moiety are also important binding structures for αHb.

From our data it can be concluded that the hydrophobic moiety of the endotoxins interacts with hydrophobic domains of αHb, consistent with previous speculations (40). This interaction leads to disaggregation of the large endotoxin aggregates (11, 36), which in turn results in better accessibility of the binding and recognition groups (epitopes) of the endotoxins to target structures. Explicitly shown by the x-ray diffraction data (Fig. 4), in the large multilamellar (L) stacks found in the absence of αHb, the epitopes are buried, to a large extent, between neighboring bilayers, whereas the reaggregation into cubic (Q) structures as a result of αHb binding leads to smaller aggregates and possibly also to more accessible epitopes because the cubic structures have large water-filled channels pervading the network, e.g. cubic structures Q24 (symmetry Pn3m; Ref. 38). These conclusions are supported by the previous electron microscopic demonstration that Hb induced disaggregation of LPS, with production of much smaller particles of LPS (14) (it should be noted that, with typical electron microscopic investigations, a differentiation of the aggregate structures is not possible).

It could be speculated that, after disaggregation of LPS, monomers might be the decisive biological units. In former papers the question of the “biologically active unit” of endotoxins was not answered unequivocally (reviewed in Ref. 42). Recently, however, it has been shown that endotoxin aggregates are the biologically active units, which are at least 2 orders of magnitude more active than monomeric solutions (43). Regarding the detailed dependence of bioactivity on aggregate number and size of LPS, no clear data are presently available.

A model of the pLA:pLPS-αHb interaction corresponding to our observations is provided in Fig. 9. As shown in the figure, the alteration of the aggregate structures is accompanied with a change in membrane curvature from zero to negative values (concave curvature; only one LPS bilayer is shown). It can be deduced from the model that the epitopes in the endotoxins are more accessible to serum and membrane proteins such as lipopolysaccharide-binding protein (LBP) and CD14 after binding to αHb, as far as the water-filled channels in the cubic structures allow the penetration of the proteins. The decisive change, however, may be the intercalation of the LPS-αHb complex into target cell membranes (see below).

For a further understanding of the change in bioactivity, the mechanisms of interactions of endotoxins with target cells should be considered. It has been shown that LBP may transport endotoxins to the cell receptor CD14 (44, 45) or directly into the cell membrane (46). LBP has also been found to exist in membrane-bound form in which it contributes to an enhancement of LPS activity (47). Subsequently, a signal is transferred in a still unknown way to membrane signaling proteins such as the Toll-like receptors (48, 49) or the ion channel MaxiK (50, 51), which have been proposed to be responsible for signaling into the cell interior. Of course, a direct interaction of endotoxin with the signaling proteins may also take place,
although direct experimental evidence for this has not yet been published. We have proposed earlier that agonistically as well as antagonistically active endotoxins may intercalate into target cell membranes such as those of mononuclear cells, by the action of LBP and CD14 (32). In the cell membrane, only LPS, with a conically shaped lipid A moiety corresponding to a nonlamellar cubic aggregate structure, within a cylindrically shaped phospholipid host matrix, provides a strong disturbance. Thus, it can induce mechanical sterical stress at the site of a signaling protein, leading to a conformational change of the protein and subsequently, to cell signaling. For example, this may hold for the MaxiK K<sup>H11001</sup> channel, which has been shown to be stress-sensitive (52). Lipid A with a cylindrical shape, corresponding to lamellar aggregate structures, does not induce sterical stress, but may inhibit the action of agonistically active LPS by blocking the binding sites. Furthermore, the endotoxin:αHb complex can intercalate into target membranes of immunocompetent cells, thus enabling cell activation. The sizes of αHb and Lipid ALPS are not to scale.

Comparison of the present results with those described in our recent paper (6) on the interaction of Hb with hexaacyl enterobacterial LPS Re and lipid A reveals a very similar behavior, i.e. acyl chain rigidification, partially non-electrostatic binding, increase in diglucosamine tilt angle, and a predominantly α-helical ααHb protein structure, which changes only slightly in the presence of LPS. The aggregate structure of a hexaacyl lipid A is, however, a priori cubic, and is converted into another cubic phase, which interestingly is also of space group Q<sup>224</sup>, as found here for the pLA:ααHb complex. Thus, ααHb appears to be an endotoxicity-enhancing agent in a general sense, as previously reported (11–13, 36, 37, 54, 55).

Beyond these similarities, however, pentaacyl endotoxin:Hb interactions per se are of considerable interest, because it is known that in nature many Gram-negative species synthesize LPS with a pentaacyl lipid A moiety. To this belong species such as Neisseria and Pseudomonas (4), which are two important causes of clinically significant infection, and which could enhance their biological activity in the presence of Hb. This might be, for example, highly relevant in the case of blood transfusion or administration of hemoglobin solutions, currently under development as red blood cell substitutes.

It is interesting to note that Hb is not the only agent capable of LBP and/or CD14 (46). Similarly, as observed here, Gorbenko (53) observed with FRET a 4.3-nm penetration of native Hb into phospholipid membranes, which corresponds to nearly full insertion of the Hb molecule into the interior of the membrane.

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of causing endotoxicity when added to an inactive lipid A. Thieblemont et al. (56) have observed that mononuclear cells were activated to produce interleukins when the antipsychotic drug chlorpromazine was added to an antagonistic lipid A or LPS. We have found (50) that this enhancement was caused by conversion of the antagonistic lipid A (again a pentaacyl lipid A from E. coli) to a lamellar into a cubic aggregate structure similar to the change produced here by ααHb. This was interpreted as resulting from an increase in the hydrophobic bulk caused by chlorpromazine, thus favoring nonlamellar structures. Based on this interpretation, it might be deduced that the binding of ααHb to LPS is also predominantly of a hydrophobic nature. However, the interaction of ααHb with LPS is more complex than that of chlorpromazine with LPS, the former also involving electrostatic interaction (Fig. 3) rather than a pure increase of the hydrophobic moiety.

The presented data should also be seen in light of the findings of Kaca et al. (12), who demonstrated with different forms of LPS and lipid A that the ester-linked fatty acids may play a role in the biologically enhancing effect of Hb and to a lesser degree also the phosphate and 2-keto-3-deoxyoctonate residues. Our data additionally indicate an influence of the negative charges (phosphate) in the lipid A in the binding process as well as (considering the phase transition data, shown in Figs. 1 and 2) the role of the hydrophobic moiety, although the lack of a change in phase transition enthalpy (Fig. 2B) rules out the production of a marked alteration of the hydrophobic moiety of lipid A caused by ααHb binding.

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