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The Charge of Endotoxin Molecules Influences Their Conformation and IL-6-Inducing Capacity[1]

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The activation of cells by endotoxin (LPS) is one of the early host responses to infections with Gram-negative bacteria. The lipid A part of LPS molecules is known to represent the endotoxic principle; however, the specific requirements for the expression of biologic activity are still not fully understood. We previously found that a specific molecular conformation (endotoxic conformation) is a prerequisite for lipid A to be biologically active. In this study, we have investigated the interdependence of molecular charge and conformation of natural and chemically modified LPS and lipid A and its transport and intercalation into phospholipid membranes mediated by human LPS-binding protein, as well as IL-6 production after stimulation of whole blood or PBMCs. We found that the number, nature, and location of negative charges strongly modulate the molecular conformation of endotoxin. In addition, the LPS-binding protein-mediated transport of LPS into phospholipid membranes depends on the presence of net negative charge, yet charge is only a necessary, but not a sufficient, prerequisite for transport and intercalation. The biologic activity is determined mainly by the molecular conformation: only conical molecules are highly biologically active, whereas cylindrical ones are largely inactive. We could demonstrate that the net negative charge of the lipid A component and its distribution within the hydrophilic headgroup strongly influence the molecular conformation and, therefore, also the biologic activity. ***The Journal of Immunology,*** **1998, 161: 5464–5471.**

Lipopolysaccharides as major amphiphilic components of the outer leaflet of the outer membrane of Gram-negative bacteria exert in isolated form a variety of biologic activities in mammals and are, thus, called endotoxins (1). Chemically, LPS consist of a hydrophilic heteropolysaccharide that is covalently linked to a hydrophobic lipid portion, termed lipid A, which anchors the molecule to the outer leaflet of the outer membrane. Because free lipid A has been shown to be responsible for the biologic activity of LPS in most in vitro and in vivo test systems, it has been termed the endotoxic principle of endotoxin (2). The specific requirements for endotoxin to be biologically active are still only partly defined. For the full expression of biologic activity, lipid A must possess a particular chemical composition and primary structure like that found in enterobacterial strains. Thus, for example, lipid A from the biologically most potent LPS of the deep rough (Re) mutant strain *Escherichia coli* F515 consists of a β-1,6-linked D-glucosamine (GlcN)[3] disaccharide carrying two negatively charged phosphates and six saturated fatty acids in a defined asymmetric distribution, four at the GlcN II and two at the GlcN I. Variation of this composition, such as a reduction of the number of charges or the number of acyl chains or a change in their distribution or degree of saturation, results in a dramatic reduction of biologic activity (3–5). These observations could be interpreted to indicate that a variation of the primary structure of endotoxin molecules influences their physicochemical behavior.

Endotoxins are amphiphilic molecules, and therefore tend to form multimeric aggregates above a critical aggregate concentration (CAC) that depends on their hydrophobicity. Above this concentration, there is an equilibrium between free monomers and aggregates. The CAC, which determines the number of free monomers, is supposed to be influenced by the amount of sugars linked to the lipid A portion of LPS as well as by the number and distribution of charges. Thus, the CAC should be highest for S-form LPS of wild-type strains, with high amounts of sugars represented by the O-specific chain, decreasing with the amount of sugars in LPS from rough mutant and deep-rough mutant strains, and be lowest for free lipid A.

The structure of the aggregates formed above the CAC can be either nonlamellar inverted (cubic, Q, or hexagonal, H_{II}) or lamellar, depending on the conformation (shape) of the contributing molecules (6). From the aggregate structure, the conformation of the individual molecules can be deduced: it is conical or wedge-shaped, with the hydrophobic moiety adopting a higher cross-section than the hydrophilic in the case of nonlamellar inverted and

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[3] Abbreviations used in this paper: GlcN, D-glucosamine; CAC, critical aggregate concentration; H_{II}, hexagonal II; Kdo, 2-keto-3-deoxyoctonate; LBP, LPS-binding protein; MALDI-TOF, matrix-assisted laser desorptional ionization time of flight; MTT, 3-(4, 5-dimethyltiazol-2yl)-2,5-diphenyltetrazoliumbromide; NBD, *N*-(7-nitrobenz-2-oxa-1, 3-diazol-4yl); $PL_{M\Phi}$, phospholipid mixture resembling the lipid composition of a macrophage membrane; Q, cubic; RET, resonance energy transfer.

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cylindrical, with the respective cross-sections being nearly identical in the case of lamellar aggregate structures. We have shown previously that a particular endotoxic conformation of the lipid A portion of endotoxin is a prerequisite for the expression of endotoxic activity. Thus, only those endotoxins having a lipid A portion that adopts a nonlamellar H_{II} or cubic aggregate structure in the isolated form, i.e., having a conical conformation, were biologically highly active (7, 8).

Several mechanisms of interaction of endotoxin with host cells have been proposed. There is experimental evidence that endotoxin molecules, similar to other amphiphilic molecules, interact unspecifically with target membranes via hydrophobic interaction. This process may cause a direct intercalation of endotoxin monomers or small aggregates into the phospholipid matrix of host cells (9), leading to either activation or neutralization. Another mechanism of interaction has been described to proceed via the specific binding of LPS to a membrane-bound receptor protein, the differentiation Ag CD14 of myelomonocytic cells, or via binding to the serum protein LPS-binding protein (LBP) and the endotoxin/LBP complex then binding to CD14 (10–14). In case of CD14-negative endothelial and epithelial cells, a soluble form of CD14 has been shown to mediate cellular activation by LPS (15, 16). There are also several investigations showing a CD14-independent cell activation, in particular at high endotoxin concentrations (17–20), but the details of this activation pathway are largely unknown. A CD14-independent, LBP-mediated transport of endotoxin molecules from aggregates directly into phospholipid membranes could recently be elucidated (21). Thus, LBP might act as a shuttle, causing a disaggregation of endotoxin aggregates by transporting the molecules directly or indirectly to target cell membranes. We and others could show that the transport activity of LBP is not restricted to endotoxin, but is also valid for negatively charged phospholipids (21, 22). Nevertheless, the role of charges for this activity of LBP remains to be defined. Independent of the detailed mechanism, however, it may be expected that the unspecific intercalation as well as the specific binding of LPS should depend on physical parameters like CAC, size, and stability of endotoxin aggregates, and the molecular conformation of the molecules. Many investigations have been reported on the interaction of LPS with CD14 and its amplifiying role in host cell activation (23–26), but only very limited data are available on the direct interaction of LPS with the target cell membrane (27–29).

In this work, we report on an approach to more closely characterize the interaction mechanism of endotoxin with target cell membranes, in particular the role of charges. The experiments are mainly focused on lipid A as the endotoxic principle and lipid A derivatives differing in the number, location, and nature of negative charges. LPS Re of *E. coli* and its dephosphorylated derivative were also included for a further variation of charges in the direct vicinity of lipid A. Except LPS Re of *E. coli*, none of the endotoxin derivatives used in these investigations are generated by intact, living bacteria. They are merely applied to obtain insight into the principles of endotoxin recognition, transport, intercalation, conformation, and biologic activity. The influence of the variations in molecular charge on the molecular conformation was derived from synchrotron small-angle x-ray diffraction data of the supramolecular structure of endotoxin aggregates in aqueous dispersion. Their influence on the LBP-mediated transport was investigated by determining the intercalation of endotoxin molecules into phospholipid liposomes resembling in their lipid composition the cytoplasmic membrane of macrophages by resonance energy transfer spectroscopy (RET). The biologic activity of the samples was assessed from the determination of the IL-6 production of endotoxin-stimulated PBMCs or ex vivo whole blood.

We found a strong modulating effect of the number, location, and nature of charges of endotoxin molecules on the molecular conformation, the interaction with LBP, and the biologic activity. According to our findings, the presence of a net negative charge is a necessary, but not a sufficient, prerequisite for the LBP-mediated transport and intercalation of endotoxin into phospholipid membranes. For the expression of biologic activity, besides a net negative charge, a particular chemical structure and endotoxic conformation are essential prerequisites.

Materials and Methods

LPS and lipid A

Bacteria from the different strains were cultured under standard conditions usually at 37°C, for the phototropic strain *Rhodospirillum fulvum* (DSM117 obtained from the Deutsche Stammsammlung von Mikroorganismen, Braunschweig, Germany) in light (100 lx) at 30°C, and LPS was extracted from the phenol-killed bacteria. The LPS of the deep rough (Re) mutant of *E. coli* strain F515 were obtained by a modified PCP (30) procedure (PCP I, i.e., phenol/chloroform/petroleum ether 2:5:8 vol %), those from the smooth strain *Salmonella enterica* serovar Friedenau were obtained by the phenol/water extraction method (31). For the phototropic strain *R. fulvum*, phenol/water extraction was followed by ultracentrifugation at 105,000 × *g* for 4 h. The LPS in the water phase was subsequently enzyme treated with proteinase K, RNase, and DNase (Sigma, Deisenhofen, Germany; and Boehringer Mannheim, Mannheim, Germany), and further purified by PCP extraction (PCP II, i.e., phenol/chloroform/petroleum ether 5:5:8 vol %). The resulting LPS were lyophilized, and are thus present in their natural salt form.

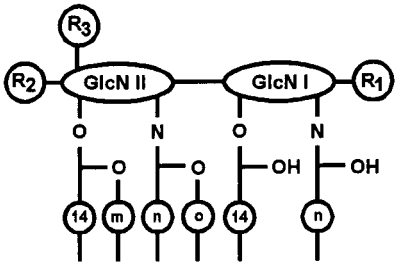
For the preparation of dephosphorylated LPS (dephosphorylated LPS Re), 750 mg LPS Re from *E. coli* strain F515 was suspended in 20 ml aqueous hydrofluoric acid (48%) for 3 days at 4°C under stirring. The reaction mixture was dialyzed extensively three times against 5 L water and lyophilized, giving 600 mg crude dephosphorylated LPS. One aliquot (390 mg) of this dephosphorylated LPS was purified on 10 preparative layer plates (2 mm, Kieselgel 60 from Merck, Darmstadt, Germany) and developed with chloroform:methanol:water (100:87.5:22.5 v/v). The resulting fractions could be visualized by dipping the dried plates into water and recovered by scraping off the white opalescent bands. Fractions with an R_f value ~0.5 in the analytical TLC (same solvent) were found to be the major product and collected (90 mg). For final purification, the dephosphorylated LPS was rechromatographed in three successive runs on one preparative layer plate using chloroform:methanol:water (100:55:7 v/v). Pure dephosphorylated LPS was visualized as stated above, expressing a single distinct band that, after elution from the plates, could be obtained in a satisfactory yield (27 mg). The resulting purified dephosphorylated LPS was analyzed with a MALDI-TOF mass spectrometer (Reflex II; Bruker, Karlsruhe, Germany) as well as ^{1}H and ^{13}C NMR (600 MHz Avance DRX; Bruker) and gave spectra fully compatible with phosphate-free LPS Re (molecular mass of $[M^{+}Na]^{+} = 2098$ Da).

Lipid A was obtained from LPS Re of *E. coli* F515 or from LPS of *R. fulvum* DSM 117 either by sodium acetate buffer (0.1 M, pH 4.4, 100°C for 1 h) or by HCl (0.1 M, 100°C for 0.5 h) treatment, purified, and converted to the triethylamine salt. The resulting preparations differ in their degree of 2-keto-3-deoxyoctonate (Kdo) and phosphate cleavage; in the acetate buffer treatment, Kdo is cleaved to 90–95%, whereas with HCl, Kdo is removed quantitatively, as well as the 1-phosphate. Thus, by the latter procedure, it is possible to obtain monophosphoryl lipid A. As shown by MALDI-TOF mass spectrometry, both the bis- and the monophosphoryl lipid A were mainly hexaacylated (~85%) and contained only minor fractions of penta- and tetraacylated structures (~10 and ~5%, respectively). Both compounds were used without further purification. As phosphate-free lipid A, the hexaacyl *E. coli*-lipid A analog (compound 503), synthesized as described earlier (32), was used.

The LPS Re of *E. coli* was in the natural salt form, and all other preparations were converted to the triethylamine salt form, except compound 503, which is uncharged and does not contain counterions.

The deacylated backbone of lipid A was derived from Re LPS of *S. enterica* serovar Minnesota strain R595, as described (33). Briefly, lipid A was de-*O*-acylated with hydrazine and subsequently de-*N*-acylated with KOH (4 M, 120°C for 16 h), followed by neutralization with HCl (4 M, 0°C). The free fatty acids were extracted with CH_2Cl_2, and the water phase was purified by gel-permeation chromatography and lyophilized.

All LPS and lipid A samples were analyzed chemically with respect to the amount of phosphate, Kdo, and GlcN with standard procedures (34), and also the content of fatty acid residues was monitored by MALDI-TOF



Sample	Nature of			Number of carbon atoms			Number of negative molecular charges
	R_1	R_2	R_3	m	n	o	
LPS Re of *E.coli*	P	P	Kdo_2	14	14	12	4
Dephosphorylated LPS Re of *E.coli*	H	H	Kdo_2	14	14	12	2
Lipid A from LPS Re of *E.coli*	P	P	H	14	14	12	2
Monophosphoryl lipid A from LPS Re of *E.coli*	H	P	H	14	14	12	1
Lipid A from LPS of *Rs. fulvum*	GalA	Hep	H	12	16	16	1
Phosphate-free lipid A (synthetic compound '503')	H	H	H	14	14	12	0

FIGURE 1. Chemical structures and number of net negative molecular charges of the investigated endotoxin samples. GalA, galacturonic acid; Hep, heptose; P, phosphate.

mass spectrometry. Only those batches that were identical to the known chemical structures were used.

The chemical structures of the various LPS and lipid A samples are schematically shown in Fig. 1. Lipid A from LPS of *R. fulvum* has, instead of phosphate groups, a galacturonic acid in 1 position and a D-*manno*-heptose in the 4′ position (35). The main variations of the chemical structures used in this study and shown in Fig. 1 consist in the number and distribution of negative charges, the former ranging from four for LPS Re to two for dephosphorylated LPS Re and lipid A from LPS Re, one for monophosphoryl lipid A from LPS Re and lipid A from LPS of *R. fulvum* to none for the phosphate-free lipid A.

All lipid A and LPS samples were prepared as aqueous suspensions. The lipids were suspended in PBS by vortexing thoroughly and were temperature cycled at least twice between 4 and 70°C, each cycle followed by intense vortexing for a few minutes, and then stored at 4°C at least 12 h before measurement. For biologic experiments, stock solutions of 1 mg/ml were aliquoted and stored at −20°C until use. The stock solutions were diluted to obtain final concentrations of 1 μg/ml to 0.1 pg/ml (corresponding to approximately 1 μM to 0.1 pM). For infrared measurements, LPS suspensions were used at approximately 20 mM (95% water content), and for x-ray diffraction at approximately 65–80 mM (85% water content).

Phospholipids

Bovine brain 3-*sn*-phosphatidylserine, egg 3-*sn*-phosphatidylcholine, and sphingomyelin from bovine brain were obtained from Sigma. 3-*sn*-phosphatidylethanolamine from *E. coli* was from Avanti Polar Lipids (Birmingham, AL). For preparation of liposomes from the phospholipid mixture resembling the composition of the cell membrane of macrophages ($PL_{M\phi}$), 3-*sn*-phosphatidylcholine, 3-*sn*-phosphatidylserine, 3-*sn*-phosphatidylethanolamine, and sphingomyelin in a molar ratio of 1:0.4:0.7:0.5 (36), the lipids were solubilized in chloroform, the solvent was evaporated under a stream of nitrogen, and the lipids were resuspended in the appropriate volume of PBS and further treated as described for LPS.

X-ray diffraction

X-ray diffraction measurements were performed as described (37) at the European Molecular Biology Laboratory outstation at the Deutsches Elektronen Synchrotron (DESY) in Hamburg using the double-focusing monochromator-mirror camera X33 (38). In the diffraction patterns presented in this work, the logarithm of the diffraction intensity log I is plotted vs the scattering vector s ($s = 2\sin\Theta/n\lambda$; 2Θ, scattering angle; $\lambda = 0.15$ nm, wavelength), and the x-ray spectra were evaluated as described previously (37, 39). Briefly, from the spacing ratios of the diffraction maxima, an assignment to defined three-dimensional aggregate structures is possible, i.e., to lamellar, nonlamellar cubic, and inverted hexagonal II. From this, the conformation of the individual molecules can be approximated (8, 40), which is cylindrical in the case of lamellar structures (the cross-sections of the hydrophilic and hydrophobic moieties are identical) and conical or wedge-shaped in the case of nonlamellar cubic and inverted H_{II} structures (the cross-section of the hydrophobic portion is higher than that of the hydrophilic one).

RET spectroscopy

The RET assay was performed as described earlier (21). Briefly, phospholipid liposomes $PL_{M\phi}$ were double labeled with the fluorescent dyes *N*-(7-nitrobenz-2-oxa-1,3-diazol-4yl)-phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (Rh-PE) (Molecular Probes, Eugene, OR). Intercalation of unlabeled molecules into the double-labeled liposomes leads to probe dilution, and with that to a decrease in the efficiency of RET: the emission intensity of the donor increases and that of the acceptor decreases (for the sake of clarity, only the donor emission intensity is shown in this work). The double-labeled $PL_{M\phi}$ liposomes were preincubated with unlabeled LPS, and human rLBP (41) was added. For a calibration of the magnitude of the intercalation of the endotoxins into the phospholipid liposomes, common preparations of $PL_{M\phi}$ and endotoxins at given molar ratios were prepared by dissolving them, together with constant amounts of the dyes, in chloroform, evaporating the solvent under a stream of nitrogen, resuspending the lipid mixture in buffer, and recording the donor emission intensity.

IL-6 induction

For the isolation of PBMCs, blood was taken from healthy donors and heparinized (20 IE/ml). The heparinized blood was mixed with an equal volume of HBSS and centrifuged on Ficoll density gradient for 40 min (21°C, 500 × *g*). The interphase layer of PBMCs was collected and washed three times 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 heparinized whole blood or PBMCs (5×10^6 N/ml) were filled into 96-well culture plates. Whole blood or PBMCs in serum-free medium were stimulated with the endotoxins. The stimuli were serially diluted in RPMI 1640 and added to the cultures at 20 μl/well. The cultures were incubated for 24 h at 37°C and 5% CO_2. Supernatants were collected after centrifugation of the culture plates for 10 min at 400 × *g* and stored at −20°C until determination of cytokine content.

For IL-6 determination, the murine cell line 7TD1 was applied (42). The culture supernatants or human rIL-6 as standard (10 ng/ml) were diluted serially in 1:4 steps in microtiter plates in 50 μl 7TD1 medium (DMEM plus 4.5 g/L glucose, 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cells were washed twice (10 min, 300 × *g*) in 7TD1 medium, and adjusted to 8×10^4 cells/ml. An aliquot (50 μl) of this cell suspension was incubated with the supernatants or rIL-6 as standard, respectively, and incubated 72 h at 37°C in 5% CO_2-containing atmosphere. For the determination of the cell proliferation, the mitochondrial reduction of 3-(4, 5-dimethyltiazol-2yl)-2,5-diphenyltetrazoliumbromide (MTT) to formazane was measured. Briefly, 10 μl MTT (5 mg/ml in PBS) was added to the cells that were incubated for 4 h with subsequent addition of 100 μl SDS solution (5% in 50% dimethylformamide). After 2-h incubation at room temperature, the absorbance at 550 nm was measured in an ELISA plate photometer. The absolute IL-6 content was determined with respect to the standards.

Results

Supramolecular structures and molecular conformations

The structure of endotoxin aggregates in aqueous dispersions is primarily determined by the conformation of the monomers. Therefore, the conformation of the monomers can be derived from the supramolecular aggregate structure determined by x-ray diffraction. The x-ray diffraction patterns of LPS Re (Fig. 2*A*) and bisphosphoryl lipid A from LPS Re (Fig. 2*C*) are characteristic of nonlamellar cubic structures with different symmetries that are frequently observed for enterobacterial lipid A and for LPS Re at high water content and in the absence of divalent cations. This can be

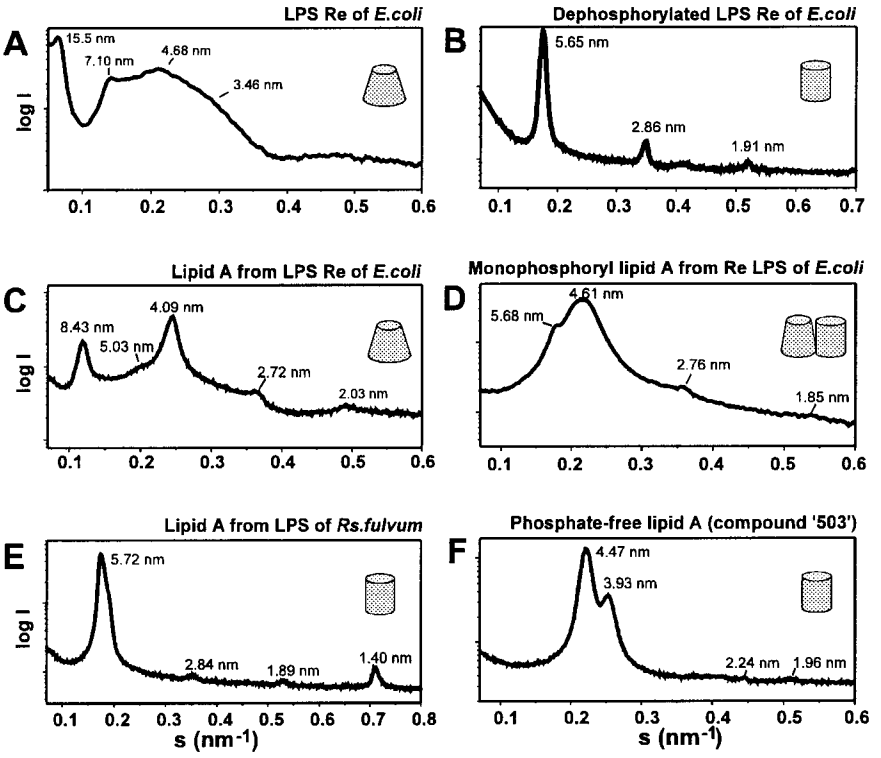


FIGURE 2. X-ray diffraction patterns of endotoxin samples. X-ray diffraction measurements of the various endotoxin samples were performed in 85% buffer at 40°C. The numbers listed in the single plots are the spacing ratios of the reflections d = 1/s (s, scattering vector). The molecular shapes of the endotoxin molecules deduced from the structural preference are shown schematically in the inserts.

deduced from the positions of the diffraction maxima that are grouped in spacing ratios corresponding to cubic structures of space group Q^{212} (LPS Re) and Q^{224} (lipid A from LPS Re). The pattern of monophosphoryl lipid A from LPS Re (Fig. 2*D*) indicates a superposition of a multilamellar with a nonlamellar structure. However, the latter is not resolvable in a straightforward way. Dephosphorylated LPS Re (Fig. 2*B*) as well as lipid A from *R. fulvum* (Fig. 2*E*) unequivocally adopt (multi)lamellar structures characterized by sharp reflections at equidistant spacing ratios. The diffraction patterns of phosphate-free lipid A (synthetic compound 503, Fig. 2*F*) indicate the existence of a normal and an interdigitated bilayer structure.

From these results, the molecular conformations of the endotoxin molecules at 37°C can be assumed to be conical for LPS Re and lipid A from LPS Re with a slightly higher cross-section of the hydrophobic as compared with the hydrophilic moiety; a mixture of both, cylindrical and conical, for monophosphoryl lipid A from LPS Re; and cylindrical for dephosphorylated LPS Re, lipid A from LPS of *R. fulvum*, and phosphate-free lipid A. However, for dephosphorylated LPS Re at temperatures >40°C, a superposition of the basically lamellar structure with a H_{II} structure was observed (data not shown), indicating a tendency of this endotoxin to adopt nonlamellar structures that are already present, but not detectable, at 40°C.

LBP-mediated transport of endotoxins into phospholipid membranes

RET spectroscopy was used to investigate the ability of LBP to transport the various endotoxins into a phospholipid membrane corresponding to the composition of the macrophage cell membrane ($PL_{M\phi}$). The results are shown in Fig. 3. It can clearly be seen that addition of LBP induces an increase of the NBD fluorescence intensity in $PL_{M\phi}$ membranes for all phosphate- or carboxylate-bearing endotoxins. The main change in NBD-fluorescence intensity occurs within about 10 s, followed by a longer period (some minutes) with an only gradually increasing intensity. These observations can be interpreted to result from an intercalation of endotoxin molecules into the liposomal membrane. In contrast, the intercalation of lipid A from LPS of *R. fulvum* carrying one net negative charge and of phosphate-free and thus neutral lipid A into the liposomal membrane is negligible.

To exclude a mere charge-driven adsorption of the endotoxin molecules to the liposomal membrane surface without intercalation to be responsible for the observed intensity increases, the interaction of a deacylated diglucosamine lipid A backbone with

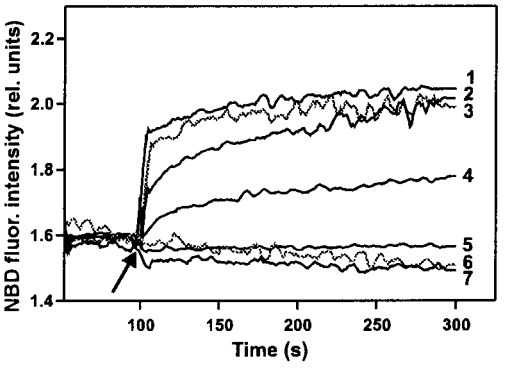


FIGURE 3. LBP-mediated transport of endotoxin samples into phospholipid liposomes. Plotted is the dependence of NBD fluorescence intensity on time for various endotoxin samples and a deacylated lipid A backbone. Double-labeled $PL_{M\phi}$ liposomes (0.1 mM) composed of a lipid mixture resembling the lipid matrix of mononuclear cells and endotoxin aggregates (0.1 mM) were preincubated, and after 100 s 50 μg/ml LBP was added (indicated by the arrow): 1, lipid A from LPS Re of *E. coli*; 2, LPS Re of *E. coli*; 3, dephosphorylated LPS Re of *E. coli*; 4, monophosphoryl lipid A from LPS Re of *E. coli*; 5, phosphate-free lipid A (synthetic compound 503); 6, lipid A from LPS of *R. fulvum*; 7, deacylated lipid A backbone from Re LPS of *S. enterica* serovar Minnesota strain R595. (Data are corrected for endotoxin-specific sensitivity differences of the RET assay by calibration with common preparations of $PL_{M\phi}$ and the different endotoxins at a fixed molar ratio.)

$PL_{M\Phi}$ membranes was also examined. In this case, no increase in fluorescence intensity, but a small decrease due to dilution of the suspension, was observed.

From a comparison of the intercalation behavior of the different compounds, it may be concluded that there is no direct correlation of the efficiency of LBP-mediated transport into the liposomal membrane to the number of negative charges. Interestingly, lipid A from LPS of *R. fulvum*, in which the 1 and 4′ phosphates are substituted by a heptose and a galacturonic acid, respectively, is intercalated into the $PL_{M\Phi}$ membrane only to a very low degree despite its effective negative charge. The effective charge of the carboxyl group of this lipid A was determined by comparing the infrared spectrum in the 1500–1400 cm^{-1} range comprising the scissoring mode of the methylene groups (at 1467 cm^{-1}) and the stretching vibration of the $\nu_s(COO^-)$ group (at 1415–1427 cm^{-1}) with that of LPS Re (two negatively charged carboxyl groups) and of lipid A from LPS Re (no carboxyl groups). The spectra were very similar for lipid A from LPS of *R. fulvum* and for LPS Re, exhibiting an intense absorption band corresponding to $\nu_s(COO^-)$ that was completely absent in the lipid A isolated from LPS Re (data not shown). Thus, lipid A from LPS of *R. fulvum* can be assumed to be present in the fully dissociated form, i.e., the carboxyl group carries one net negative charge.

IL-6 induction in whole blood and PBMCs

As a measure for the biologic activity of the lipid A and LPS samples, their ability to induce IL-6 in ex vivo whole human blood was determined. IL-6 is, besides IL-1 and TNF-α, one of the important mediators induced by endotoxin. The results are shown in Fig. 4*A* for endotoxin concentrations in the range 0.001–1000 ng/ml and are compared with the data obtained for LPS from *Salmonella friedenau* (S-form LPS) used as a calibration standard. Clearly, LPS Re has the strongest IL-6-inducing capacity, which is nearly identical to that of LPS S-form from *S. friedenau*. Lipid A from LPS Re is, in the medium concentration range 0.1–10 ng/ml, one order of magnituide less active, followed by the monophosphoryl lipid A and dephosphorylated LPS Re, which are nearly three orders of magnitude less active. Lipid A from LPS of *R. fulvum* displays very weak activity. The synthetic phosphate-free lipid A is completely inactive.

The IL-6-inducing capacity of LPS Re, dephosphorylated LPS Re, and lipid A with and without the phosphate groups, respectively, was measured also with PBMCs in the absence of serum, i.e., in particular in the absence of LBP. The results, given in Fig. 4*B*, show that the reference LPS S-form from *S. friedenau* and LPS Re behaves in a way similar to that in the whole blood assay regarding the amount of induced IL-6 as well as the lowest endotoxin concentration required to provoke a measurable effect (~0.01 ng/ml). However, in the serum-free system a steep increase in IL-6 production at endotoxin concentrations in the range 0.01–0.1 ng/ml and a subsequent plateau up to the highest concentrations (saturation) are observed. In contrast, in the serum-containing system there is a gradual increase over a wide range of endotoxin concentrations.

In Fig. 5, the various physicochemical parameters (molecular charge, molecular conformation, and LBP-mediated intercalation) and the biologic data (amount of endotoxin necessary to induce 1 ng/ml IL-6 in whole blood or PBMCs) are summarized to allow a direct comparison and correlation.

Discussion

In this study, the influence of charges on physicochemical properties of various clearly defined endotoxins and endotoxin derivatives and on their IL-6-inducing capacity in whole blood and in serum-free PBMCs was investigated. The number of molecular charges of the endotoxin samples ranged from zero to four. They originated from the phosphate residues on the lipid A backbone and the carboxyl groups in the Kdo region of LPS Re of *E. coli*, or from the galacturonic acid residue attached to the GlcN I in the case of lipid A from LPS of *R. fulvum* (Fig. 1). In these investigations, particular attention has been paid to a consequent variation of the chemical structure of the endotoxins. Thus, all derivatives differing in the net charge have been obtained from the same LPS batch (LPS Re of *E. coli* F515), and the synthetic compound 503 corresponds to the dephosphorylated lipid A component of this LPS. Lipid A from LPS of *R. fulvum* was used, because its only negative charge is represented by the galacturonic acid. The physical measurements comprised the determination of the supramolecular structure of endotoxin aggregates and the conformation of the molecules derived therefrom and the LBP-mediated transport of endotoxin into liposomal phospholipid membranes corresponding to the composition of the macrophage cytoplasmic membrane.

For the following discussion of the influence of the molecular charge on physicochemical properties of endotoxins and their IL-6-inducing capacity, it should be considered that the judgment of the separate influence of one of the parameters, molecular charge,

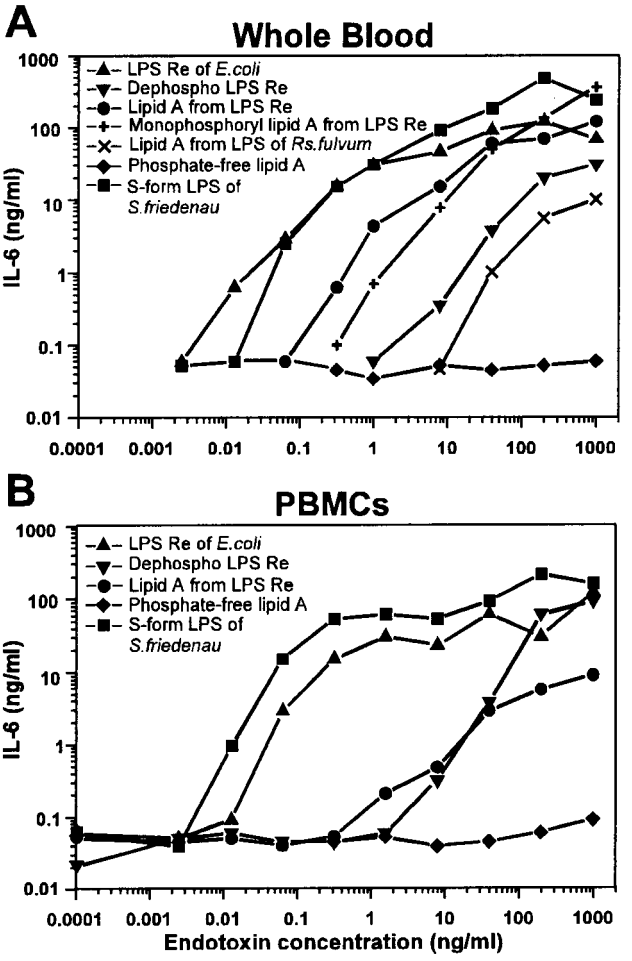


FIGURE 4. IL-6-inducing capacity of endotoxin samples. Dependence of IL-6 production on endotoxin concentration after 24-h stimulation of ex vivo whole blood (*A*) or PBMCs in the absence of serum (*B*) with various endotoxin preparations. The data are representative for at least three independent experiments.

Sample	Amount of endotoxin necessary to induce 1 ng/ml IL-6 (ng/ml)		Number of negative molecular charges	molecular conformation	LBP-mediated transport into $PL_{M\Phi}$
	in whole blood	in MNC			
LPS Re of *E.coli*	0.02	0.04	4		++
Dephosphorylated LPS Re* of *E.coli*	12	15	2		++
Lipid A from LPS Re of *E.coli*	0.4	15	2		++
Monophosphoryl lipid A from LPS Re of *E.coli*	1.2	ND	1		+
Lipid A from LPS of *Rs.fulvum*	40	ND	1		–
Phosphate-free lipid A (synthetic compound '503')	>>1000	>>1000	0		–

FIGURE 5. Correlation of IL-6 induction, number of net negative molecular charges, molecular conformation, and LBP-mediated transport of endotoxins into $PL_{M\phi}$ liposomes. The amount of endotoxin necessary to induce 1 ng/ml IL-6 in whole blood or PBMCs, respectively, is taken from Fig. 4, *A* and *B*. (★, At temperatures >40°C, the tendency of this compound to adopt nonlamellar inverted (H_{II}) structures, i.e., a conical molecular conformation, becomes significant.)

molecular conformation, and LPB-mediated intercalation, is hampered by the interdependence between them.

There is increasing evidence that the biologic activity of endotoxin is mediated by oligomeric endotoxin units down to monomers (43). Therefore, it may be assumed that the structure and the stability of endotoxin aggregates influence not only the equilibrium between aggregates and monomers, but also the interaction of endotoxin with LBP, which is supposed to shuttle endotoxin to CD14 or soluble CD14, lipoprotein particles (14, 44, 45), or phospholipid membranes (21, 22, 46). Endotoxin monomers, regardless whether existing per se or being liberated from aggregates by the action of LBP, may interact with the various endotoxin-binding proteins or may intercalate into host cell membranes, thus causing a further release of monomers from the aggregates to maintain the equilibrium, and thereby keeping the process in progress.

It was found previously that the molecular conformation adopted by the lipid A component of endotoxin molecules influences the biologic activity. Thus, highest activity was found for lipid A (and the corresponding LPS) with a conical shape, whereas samples adopting a cylindrical conformation were largely inactive (6). In some cases, the data from physical measurements were correlated with those from biologic experiments performed in other laboratories with other batches of endotoxins, the biologic systems comprising a variety of different activities such as pyrogenicity in rabbits, lethal toxicity in mice, and cytokine induction in vitro and in vivo using monocytes and endothelial cells (47–49). In the present investigations, we correlated physicochemical parameters of the same samples of clearly defined lipid A and LPS Re directly with their ability to induce IL-6 production in human ex vivo whole blood or PBMCs.

According to Fig. 2, lipid A from LPS Re of *E. coli* F515 in bound or free form adopts a nonlamellar inverted structure. Therefore, the single constituting molecules should have a conical geometry, and should thus be biologically active. Phosphate-free lipid A (compound 503) and lipid A from LPS of *R. fulvum* adopt lamellar supramolecular structures due to the cylindrical geometry of the monomers, and they should, therefore, be largely biologically inactive. The same should be expected for the lipid A component of dephosphorylated LPS Re. These predictions are confirmed by biologic assays (Fig. 4, *A* and *B*). The remaining bioactivity of dephosphorylated LPS Re can be explained by its tendency to adopt nonlamellar structures that are in fact observed at temperatures >40°C and that give evidence for the influence of the charges of the Kdo on the conformation of the lipid A moiety. From these results, it may be concluded that a conical conformation of the lipid A component with a higher cross-section of the hydrophobic than of the hydrophilic part is an important prerequisite for the induction of high IL-6 amounts.

Another interesting aspect of the present results arises from the observation that the removal of the glycosidic phosphate group of lipid A from LPS Re leads to a transition from a pure cubic into a mixed lamellar/cubic, and the removal of both phosphates (corresponding to synthetic compound 503) to a complete lamellarization with two coexisting systems of different periodicity (Fig. 2). Because the hydrophobic moiety is the same for all three samples, the formation of lamellar systems is connected with an increase of the space requirement of the hydrophilic moiety. This implies that the simple geometric consideration, that the space requirement of the hydrophilic moiety should be reduced by the removal of the phosphates, is inappropriate. Rather, the space-reducing effect of counterions bridging phosphate groups of neighboring molecules, leading to a reduction of the backbone cross-section, is in dephosphorylated or phosphate-free compounds ineffective.

The molecular charge also exerts a modifying effect on the LBP-induced transport and intercalation into liposomal membranes ($PL_{M\phi}$). This is apparent from the evaluation of the RET data (Fig. 3) that clearly indicate strong differences for endotoxins with different chemical structures. Because control experiments with the deacylated lipid A backbone consisting of a bisphosphorylated diglucosamine displayed no increase of the fluorescence signal at all, a pure adsorption or association of the endotoxin molecules to the liposome surface can be excluded. From Fig. 3, it becomes obvious that the LBP-mediated transport and intercalation into $PL_{M\phi}$ liposomes are comparable for those endotoxin structures that carry two or more negative charges (curves 1–3). From a comparison of the behavior of dephosphorylated LPS Re (curve 3) with two negatively charged carboxylate groups and free lipid A form LPS Re (curve 1) carrying two negatively charged phosphates, it could be concluded that phosphate groups are not required for the interaction of LPS with LBP. However, this simple picture is complicated by a comparison of the transport efficacy of the two endotoxins carrying one negative charge, monophosphoryl lipid A from LPS Re (curve 4), and lipid A from LPS of *R. fulvum* (curve 6), the former being intercalated into the liposomal membrane at a significantly higher rate than the latter. This implies that at least in those cases in which the only effective charge is located at the lipid A backbone, the nature of the functional groups representing the

negative charge is critical for LBP-mediated transport. Furthermore, these examples demonstrate that the nature of the functional group representing the negative charge influences the molecular conformation. Monophosphoryl lipid A from LPS Re with the only phosphate group present in 4′ position assumes a weakly developed conical geometry, whereas the lipid A from LPS of *R. fulvum*, which carries two voluminous sugars, in 1 position a galacturonic acid and in 4′ position a heptose, assumes a pure cylindrical conformation. These considerations are backed by first unpublished results on the IL-6-inducing capacity of a derivative of the synthetic *E. coli* lipid A (compound 506) in which the 1 phosphate is replaced by a carboxymethyl group. This derivative displayed activity similar to that of the corresponding bisphosphorylated lipid A in a whole blood assay. These data are, furthermore, indicative of a conical conformation of this lipid A analogue.

At least in the absence of serum proteins, the spontaneous intercalation of monomers into the macrophage membrane is likely to be part of the activation process. The extent of this intercalation should be proportional to the number of monomers available, which increases with increasing length of the hydrophilic LPS region (higher CAC). Such a dependence is impressively apparent from the data depicted in Fig. 4*B*, which show increasing IL-6-inducing capacity in the sequence phosphate-free lipid A < dephosphorylated LPS Re and lipid A from LPS Re < LPS Re < LPS S-form. In particular, the almost identical biologic activities of dephosphorylated LPS Re and lipid A from LPS Re are in agreement with this interpretation, because it seems reasonable to roughly assume a similar value for their CAC, both preparations having two negative charges in the backbone and an identical lipid component. In addition, the observation that the molecular conformation of LPS Re is identical to that of lipid A from LPS Re, and also that of dephosphorylated LPS Re is identical to that of phosphate-free lipid A, but the respective biologic activities differ, can be explained by the influence of the number of available monomers. Furthermore, the results for lipid A from LPS Re indicate that under serum-free conditions a conical conformation is a necessary but not sufficient condition for the expression of high biologic activity because cell activation is only possible with a sufficient number of monomers.

In this context, it might be interesting to discuss briefly the background of the missing biologic activity of some bisphosphorylated lipid A and lipid A part structures, in particular the lipid A from LPS of *Rhodobacter capsulatus* and lipid A precursor IVa (synthetic compound 406). For the former compound, we have determined a cylindrical conformation (40), which readily explains the lacking biologic activity, irrespective of the higher CAC value due to a reduced hydrophobicity (only five fatty acid residues at the lipid A backbone). This argumentation should hold even more for compound 406, which carries only four fatty acids.

In conclusion, our data show that in addition to a particular chemical structure such as that found in enterobacterial strains and a unique endotoxic conformation, the presence of negative charges in endotoxin molecules is a further prerequisite for biologic activity. Even though, at present, it cannot be excluded that further parameters influence endotoxin biologic activity, the influence of the various physicochemical parameters investigated in this study and their interdependences allows us to understand the sequence of the biologic activities of the various endotoxins and endotoxin derivatives.

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