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Physico-chemical analysis of lipid A fractions of lipopolysaccharide from *Erwinia carotovora* in relation to bioactivity

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Abstract

Highly purified bisphosphoryl, monophosphoryl and dephosphoryl lipids A from *Erwinia carotovora* with different acylation patterns were characterized physico-chemically. Applying matrix assisted laser desorption/ionization mass spectrometry, the purity of the lipid A fractions was determined, and from monolayer measurements the molecular space requirement was estimated. Fourier transform infrared spectroscopy allowed the elucidation of the gel to liquid crystalline phase transition of the acyl chains as well as the determination of the tilt angle of the diglucosamine backbone with respect to the acyl chain direction applying dichroitic measurements with attenuated total reflectance. With synchrotron radiation small-angle X-ray diffraction the supramolecular aggregate structure was determined, and with fluorescence resonance energy transfer spectroscopy the lipopolysaccharide binding protein induced intercalation of lipid A into a phospholipid matrix corresponding to that of the macrophage membrane was investigated. From the results, a clear dependence of the physico-chemical parameters on the particular lipid A structure can be followed. Furthermore, these parameters correlate well with the biological activities of the various lipids A as deduced from their ability to induce biological activity (*Limulus* assay and cytokine induction in mononuclear cells). These results contribute to a closer interpretation of the physico-chemical prerequisites for endotoxic activity as found for enterobacterial lipid A.

Keywords: *Erwinia carotovora*; Lipid A; Endotoxic conformation; Cytokine induction; Phase transition

1. Introduction

Lipopolysaccharide (LPS) as major amphiphilic component of the outer leaflet of the outer membrane of Gram-negative bacteria is one of the most potent inducers (endotoxin) of various biological responses in mammals such as pyrogenicity, adjuvant activity, local Shwartzman reaction, and induction of cytokines such as interleukins and tumor necrosis factor α (TNF-α) which may result in sepsis syndrome or even septic shock with fatal outcome [1]. Since the biological effects are induced by the hydrophobic anchor of LPS, termed lipid A, the latter is called the endotoxic principle of LPS. It has been observed, however, that only lipids A like those

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from enterobacteria such as *Salmonella*, and *Escherichia* induce the whole bandwidth of biological reactions [2]. These lipids A consist mainly of a bisphosphorylated diglucosamine backbone with 3-acyloxyacyl and 3-hydroxy ester- and amide-linked acyl chains.

The role of LPS and its particular chemical structure with respect to the virulence and pathogenicity of bacteria is by far not understood. Only in single cases, such as those found for *Salmonella*, the lipid A part can directly be made responsible for pathogenicity [3]. In the case of the plant pathogenic bacteria *Erwinia carotovora*, so far its pathogenicity was related to plant cell wall degrading enzymes [4]. Recently, however, it was found that a mutation in a gene required for LPS biosynthesis also affects the virulence of these bacteria [5]. Therefore, the question of the responsibility of LPS not only for endotoxicity but also for pathogenicity in a wider sense is of high scientific relevance.

We have found that the physical or physico-chemical behavior of aqueous suspensions of LPS and lipid A is important for an understanding of their biological action [6,7]. This refers (i) to the value of the critical micelle concentration (cmc), which defines the concentration above which on further addition of lipids to the bulk solution no further increase in monomer concentration takes place but aggregates are formed, (ii) to the shape of the lipid aggregates above the cmc and the shape of the constituting molecules, (iii) to the mobility of the hydrocarbon chains (fluidity) within the aggregates, and (iv) to the dependence of all these parameters on pH, concentration of mono- and divalent cations, and temperature. For LPS and lipid A from *Escherichia coli* and *Salmonella minnesota*, we have shown that under near physiological conditions physico-chemical characteristics are correlated to their ability to express biological activity [8–10].

Here, we report on a comprehensive physico-chemical analysis of highly purified mono- and bisphosphoryl hexa- to heptaacyl, in some cases pentaacyl lipid A part structures of LPS from *E. carotovora*, which are comparable only to the purity of synthetic compounds. We have found distinct differences of some investigated parameters between different part structures that might explain the considerable differences in cytokine inducing activity, and can therefore extend the understanding of the correlation of physico-chemical structure to biological activity.

2. Materials and methods

2.1. LPS and lipid A

E. carotovora FERM P-7576 was cultured in liquid medium by a two-stage cultivation method, and LPS was isolated from the lyophilized cells as reported previously [11].

The bisphosphoryl lipid A was prepared from LPS by the following procedure: LPS (100 mg) was dispersed in acetate buffer (0.1 M, pH 4.4, 10 ml) and heated on a aluminum brick heater at around 100°C for 1.5 h. The sediment (approx. 50 mg) was charged onto a precoated Silica gel 60 thin layer chromatography plate (TLC, 20 cm × 20 cm, thickness 1 mm, Merck, Darmstadt, Germany) and developed in a chloroform:methanol:water mixture (100:75:15, v/v). The lipid A fractions were visualized by dipping the TLC plate into pure water; the hydrophobic bands that appeared were scraped from the plate and the compound was separated from the silica gel using chloroform-methanol eluants. For the first elution chloroform:methanol = 2:1 (v/v) was used, mixing ratio 1:1 for the second, and 1:2 for the third elution. The eluates were combined and evaporated to dryness. The lipid A preparations were dissolved in the mixing solvent (chloroform:methanol = 2:1, v/v), and insoluble substance was removed by centrifugation and discarded. The solvent was purged by a stream of nitrogen gas until the lipid A precipitate appeared, then methanol was added to promote lipid A sedimentation. Lipid A was harvested by centrifugation, washed using methanol, dispersed in pure water, and lyophilized.

The 4′-monophosphoryl lipid A preparations were obtained by acid hydrolysis of LPS in 0.1 M sulfuric acid, and purified by TLC [12]. Dephosphorylated lipid A samples were prepared using HF treated LPS by a method described previously [9,13]. Briefly, LPS (200 mg) was suspended in 46% aqueous hydrofluoric acid (50 ml) and agitated for 120 h at 4°C. HF was removed from the sample solution by dialyzing against distilled water. The lyophilized HF treated LPS was hydrolyzed in 0.1 M sulfuric acid solution, the resulting sediment was collected and subjected to TLC purification in a developing solvent consisting of chloroform:methanol (9:1, v/v).

2.2. *Chemicals*

Bovine brain 3-*sn*-phosphatidylserine, egg 3-*sn*-phosphatidylcholine, and sphingomyelin from bovine brain were obtained from Sigma (Deisenhofen, Germany). The fluorescent dyes *N*-(nitrobenz-2-oxa-1,3-diazol-4-yl)-PE (NBD-PE) and *N*-(rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes (Eugene, OR, USA).

2.3. *Mass spectrometry*

Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) of the isolated and derivatized lipid A was performed with a Reflex III (Bruker-Franzen Analytik, Bremen, Germany) in a linear and reflector time of flight (TOF) configuration at an acceleration voltage of 20 kV and delayed ion extraction. After separation of the differently acylated lipid A components on preparative TLC plates, the samples were purified from silica gel in several washing and centrifugation steps. The compounds were then dissolved in distilled water at a concentration of 10 μg/μl. To remove disturbing cations, the sample solution (15 μl) was treated with small amounts of cation exchanger (Amberlite IR-120, Merck, Darmstadt, Germany). 2 μl of the supernatants were mixed with 2 μl 0.5 M matrix solution of 2,5-dihydroxybenzoic acid (Aldrich, Steinheim, Germany) in methanol and aliquots of 0.5 μl were deposited on a metallic sample holder and analyzed immediately after drying in a stream of air. Mass scale calibration was performed internally with similar compounds of known chemical structure.

2.4. *Molecular area of monolayers at the air-water interface*

For the determination of the molecular area, monolayer measurements at the air-water interface on a film balance (Langmuir trough) were performed. For this, 3 μl of 1 mM lipid A solubilized in chloroform:methanol (10:1, v/v) were spread on the aqueous subphase (100 mM NaCl in distilled water). After evaporation of the solvent, the pressure-area isotherms were recorded at a compression rate of 1.8 cm^2 min^{-1} at room temperature. The molecular area was determined at a lateral pressure of 30 mN m^{-1} which is assumed to resemble the value in biological bilayer membranes [14].

2.5. *$\beta \leftrightarrow \alpha$ gel to liquid crystalline phase transition of the acyl chains*

The determination of the phase behavior was done with Fourier transform infrared (FTIR) spectroscopy on an IFS55 (Bruker, Karlsruhe, Germany) using 10 mM lipid A suspensions, which were prepared by adding HEPES buffer to the appropriate amount of lipid A, heating the suspension to 60°C, vortexing it for 1 min, and recooling it to 4°C. This procedure was repeated at least twice, and the lipid A sample was cooled for 12 h prior to measurement. The peak position of the symmetric stretching vibration of the methylene groups $\nu_s(CH_2)$ was taken as the measure of acyl chain order, lying around 2849–2850 cm^{-1} in the highly ordered gel phase and 2852–2853 cm^{-1} in the less ordered liquid-crystalline phase [15,16].

2.6. *Conformational analysis of hydrated samples*

The measurements of hydrated lipid A suspensions were performed with lipid samples as indicated above. An aliquot (10 μl) was spread on a CaF_2 crystal and allowed to stand at room temperature until all free water was evaporated. After this, IR spectra were recorded at 37°C.

2.7. *Orientation measurements with attenuated total reflectance (IR-ATR)*

For the determination of the orientational behavior, attenuated total reflectance utilizing polarized IR light was used as previously introduced by Fringeli and co-workers for phospholipids [17] and in our investigation for lipid A [18]. Dichroic ratios *R*, i.e., the ratio of the peak areas of particular vibrational bands from the diglucosamine backbone at 1170, 1085, and 1045 cm^{-1}, were evaluated. These are unspecific vibrations of the sugar ring such as C-O-C ([19] and references therein). From the determination of *R*, the angle θ between the plane of the diglucosamine backbone and the direction of the hydrocarbon chains could be calculated according to the formulas given in [18].

2.8. Supramolecular aggregate structure

The three-dimensional supramolecular structures of lipid A aggregates in water systems were determined by small-angle X-ray diffraction with synchrotron radiation. For this, lipid A in appropriate concentrations (85% water content, i.e., 15 mg/85 μl buffer) was temperature-cycled as indicated above and analyzed on the double focusing monochromator-mirror camera X33 of the European Molecular Biology Laboratory outstation at the Hamburg Synchrotron Radiation Facility HASYLAB [20]. Diffraction patterns in the range of scattering vectors $0.07 < s < 1\ \text{nm}^{-1}$ ($s = 2 \sin \theta/\lambda$) were recorded using a linear detector [21]. The wavelength calibration was done with tripalmitin as standard with a periodicity of 4.06 nm. In the diffraction patterns, the logarithm of the diffracted intensity $\log I$ is plotted against s. The evaluation of the spectra was performed according to procedures described in the literature [22,23], which allow assignment of the spacing ratios of the main scattering maxima to defined the three-dimensional structures.

2.9. Incorporation of lipid A into phospholipid membranes

The ability of some selected lipid A to incorporate into target cell membranes on its own or mediated by lipopolysaccharide binding protein (LBP) was determined from fluorescence resonance energy transfer (FRET) spectroscopic measurements as described before [24]. Briefly, phospholipid liposomes corresponding to the composition of the macrophage membrane PL_M, i.e., phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin in a molar ratio of 1:0.4:0.7:0.5, were double labeled with the fluorescent dyes NBD-PE and Rh-PE. NBD-PE and Rh-PE were dissolved in chloroform and added to the lipids in the chloroform phase to a final molar ratio [PL]:[NBD-PE]:[Rh-PE] = 100:1:1. The emission wavelength of one dye, the donor (NBD-PE), is in the range of the excitation wavelength of the second dye, the acceptor (Rh-PE). The energy transfer between these two dyes is sensitive to spatial separation. Intercalation of unlabeled molecules into the double labeled liposomes leads to probe dilution and with that to a decrease in the efficiency of FRET: the emission intensity of the donor increases and that of the acceptor decreases (for the sake of clarity, here we only show the donor emission intensity). 900 μl of the double labeled $PL_{M\phi}$ liposomes (0.01 mM) at 37°C were excited at 470 nm (excitation wavelength of NBD-PE), and the fluorescence emissions of NBD-PE (531 nm) and Rh-PE (593 nm) were adjusted to yield identical intensities and recorded for 50 s under continuous stirring to determine the base line. After 50 s, unlabeled lipid A (100 μl, 0.1 mM) was added, after a further 50 s the appropriate amount of LBP was added, and the emission signals were recorded for at least another 300 s.

2.10. Determination of endotoxin activity by the chromogenic Limulus test

Endotoxin activity of hexa- and heptaacyl lipid A from *E. carotovora* as compared to control lipid A in the concentration range 10 μg/ml down to 10 pg/ml from *E. coli* F515 LPS was determined by a quantitative kinetic assay based on the reactivity of Gram-negative endotoxin with *Limulus* amebocyte lysate (LAL) [25], using test kits from BioWhittaker (No. 50-650U, Germany).

2.11. Stimulation of human mononuclear cells (MNC) by lipid A

In experiments aiming at the determination of the cytokine inducing capacity of lipid A, human mononuclear cells were stimulated with lipid A, and the TNF production of the cells was determined in the supernatant. For the isolation of MNC, heparinized (20 IU/ml) blood was taken from healthy donors and processed directly by mixing it with an equal volume of Hanks' balanced solution and centrifuging it on a Ficoll density gradient for 40 min (21°C, 500×*g*). The interphase layer of mononuclear cells was collected and washed twice in Hanks' 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 MNC (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 and 5% CO_2. Supernatants were collected after centrifugation of the culture plates for 10 min at $400\times g$ and stored at −20°C until determination of cytokine content.

The immunological determination of TNF-α in the cell supernatant was measured in a sandwich ELISA. 96-Well plates (Greiner, Solingen, Germany) were coated with a monoclonal antibody against TNF (clone 6b from Intex, 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 POD conjugated rabbit anti-rTNF antibody. The plates were shaken 16–24 h at 4°C. For the removal of free antibodies, the plates were washed six times in distilled water. Subsequently the color reaction was started by addition of tetramethylbenzidine/H_2O_2 in alcoholic solution and after 5–15 min stopped by the addition of 0.5 M sulfuric acid. In the color reaction, the substrate is cleaved enzymically, and the product was measured photometrically at a wavelength of 450 nm, and the values were related to the standard.

3. Results

3.1. Lipid A

The chemical structures of the investigated mono- and bisphosphoryl lipid A are given in Fig. 1. The yield of bisphosphoryl heptaacyl and hexaacyl lipid A was approx. 1.5% and 5% with respect to the parent LPS, respectively. Both lipid A preparations were indicated as single spot on the analytical TLC by the sulfuric acid spray visualization method. The negative ion MALDI-TOF spectra indicated for both lipids A very high purity, with mass numbers identical to those reported previously [26]. Heptaacyl lipid A yielded a major precursor ion peak $(M-H)^-$ at *m/z* 2007.8 and hexaacyl lipid A at *m/z* 1769.4 (Fig. 2). The yield of 4′-monophosphoryl lipid A from the parent LPS was around 3% for the heptaacyl and 14% for the hexaacyl derivative. MALDI-TOF-MS analysis indicated identical molecular ion peaks to that obtained by fast atom bombardment mass spectral analysis [12]. Phosphate-free lipid A preparations were obtained from natural LPS with yields of 2% and 10% for heptaacyl and hexaacyl lipid A, respectively. The positive ion MALDI-MS measurement using 2,4-dihydroxybenzophenone for the ionization matrix showed precursor ion $(M+Na)^+$ peaks at *m/z* 1872.3 (heptaacyl) and *m/z* 1633.8 (hexaacyl).

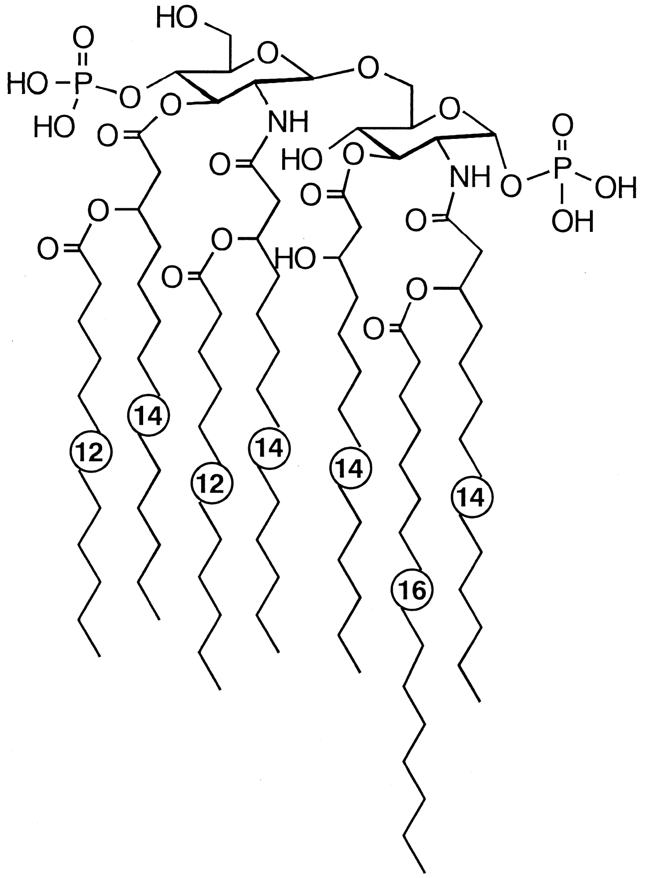

Fig. 1. Chemical structure of the bisphosphoryl heptaacyl lipid A fractions isolated from LPS of *E. carotovora* FERM P-7576. The monophosphoryl derivatives miss the phosphate group at the 1-position, and the hexaacyl compounds lack the hexadecanoic acid at the 2-position.

3.2. Monolayer isotherms

Monolayer isotherms at the air-water interface at room temperature were performed for monophosphoryl penta-, hexa-, and heptaacyl (Fig. 3A) and

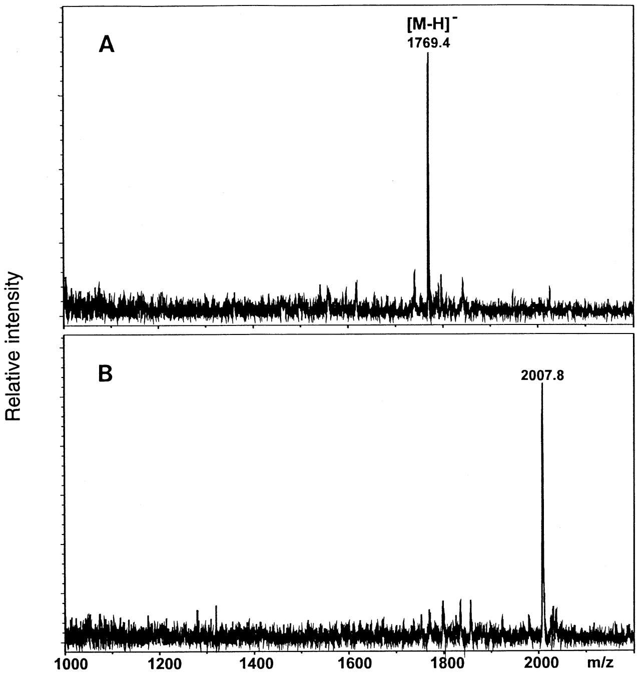

Fig. 2. Negative ion MALDI-TOF mass spectra of bisphosphoryl hexaacyl (A) and heptaacyl (B) lipid A from *E. carotovora*.

for bisphosphoryl hexa- and heptaacyl lipid A (Fig. 3B). All lipid A preparations exhibit a distinctly expressed transition from a liquid expanded into a liquid condensed phase in the pressure range 10–20 mN m^{-1}, except for the heptaacyl monophosphoryl sample with a transition around 3 mN m^{-1}, which would correspond to the highest phase transition temperature in the bilayer system.

The molecular space requirement is lower for hexaacyl (1.26 nm^2) than for heptaacyl (1.35 nm^2) bisphosphoryl lipid A. For the monophosphoryl series, there is a systematic variation of the molecular space requirement ranging from 0.89 nm^2 for the pentaacyl to over 1.16 nm^2 for the hexaacyl to 1.27 nm^2 for the heptaacyl lipid A. The standard deviation of these molecular area values can be estimated to be ±0.05 nm^2, obtained from at least five single measurements.

3.3. Gel to liquid crystalline phase transition

The gel to liquid crystalline phase transition of the acyl chains of the various lipid A preparations was determined by the analysis of the peak position of $\nu_s(CH_2)$. Beside the pure samples, in some cases also the phase transition behavior in the presence of divalent cations that are important for the stabilization of LPS in the outer membrane [27] was examined. The results for the hexaacyl monophosphoryl lipid A is given in Fig. 4A and that for hexaacyl bisphosphoryl lipid A in Fig. 4B. The position of T_c, lying at 50°C for the sample in pure water, is shifted to around 56°C in the presence of Mg^{2+}. Furthermore, the decrease of the wave numbers in both phases indicates an overall ordering of the lipid A acyl chains. Similar results are found for the other phosphorylated lipid A preparations. However, the monophosphoryl lipid A samples show only a very slight increase in T_c, whereas the overall ordering remains the same (Fig. 4A). Unexpectedly, the latter shows a significantly higher $T_c = 56°C$ than that of the bisphosphoryl lipid A. The dephosphoryl hexaacyl as well as heptaacyl lipid A, which have a

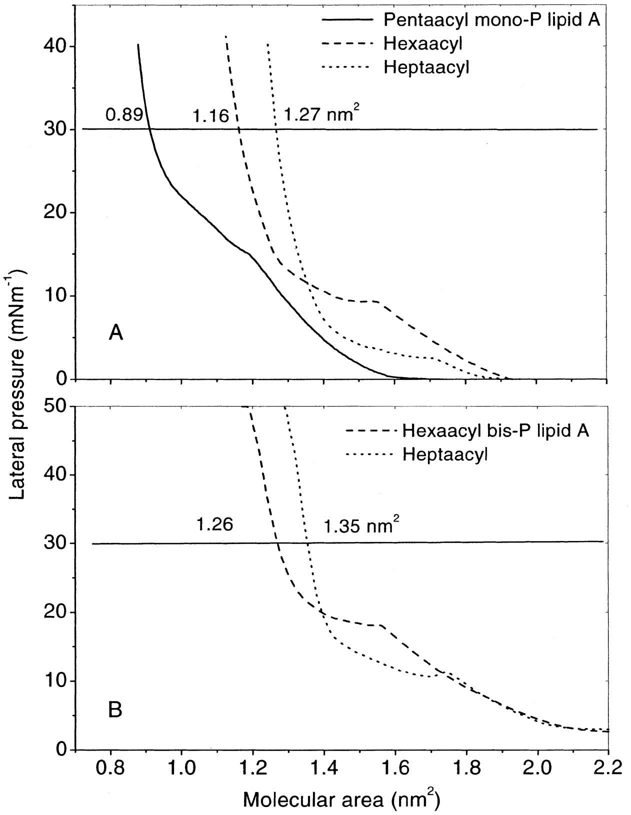

Fig. 3. Monolayer isotherms for monophosphoryl penta- through heptaacyl lipid A (A) and bisphosphoryl hexa- and heptaacyl lipid A (B). The molecular area was determined at a lateral pressure of 30 mN m^{-1}.

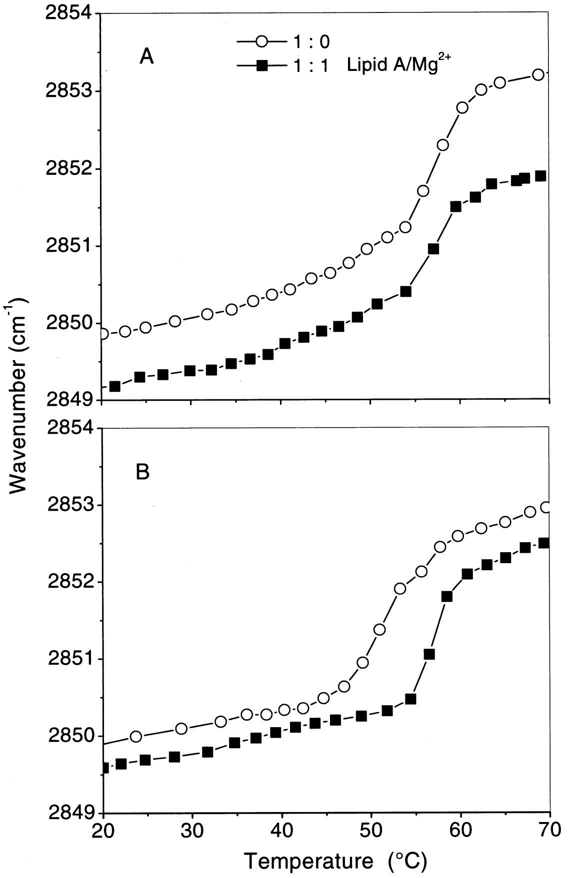

Fig. 4. Peak position of the symmetric stretching vibrational band $\nu_s(CH_2)$ versus temperature for monophosphoryl hexaacyl lipid A (A), and bisphosphoryl hexaacyl lipid A (B), in the absence and presence of Mg^{2+}.

nearly identical phase transition in the range 60–80°C, have a markedly higher state of order in the gel phase than the other lipid A compounds which can be taken from the peak position of $\nu_s(CH_2)$ lower than 2849 cm^{-1} as compared to 2849.5–2850.0 cm^{-1}, and their phase transition is not influenced by the presence of Mg^{2+} (data not shown).

3.4. Conformational analysis of the phosphate groups

For all lipids A the negatively charged phosphate band contour in the wave number range 1320–1180 cm^{-1} was analyzed infrared spectroscopically. One band component was found at 1220 cm^{-1} which corresponds to highly hydrated phosphate groups [28]. This band was only weakly expressed for monophosphoryl lipid A. Furthermore, the occurrence of an additional band component at 1257 cm^{-1}, corresponding to poorly hydrated phosphates, indicated besides reduced mobility also poor hydration of the 4′-phosphate.

3.5. Supramolecular aggregate structure

The aggregate structures of the various lipids A were investigated with synchrotron radiation small-angle X-ray diffraction at 85% water content and at different temperatures. The results are shown for the different phosphorylated hexaacyl series in Fig. 5A and for the respective heptaacyl lipid A in Fig. 5B.

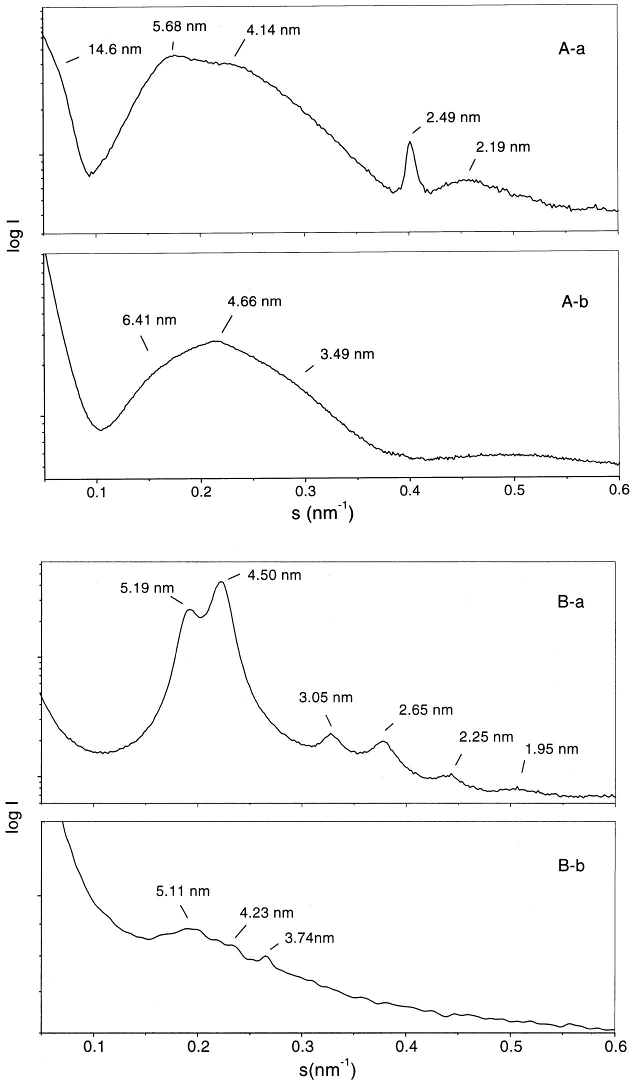

Fig. 5. Small-angle X-ray diffraction patterns for hexaacyl (A) and heptaacyl (B), for monophosphoryl (a) and bisphosphoryl (b) lipid A.

The diffraction pattern of the hexaacyl bisphosphoryl lipid A is not completely resolved; however, its shape and the location of the main reflection at 4.66 nm and the two shoulders at 6.41 nm and 3.49 nm resemble that of lipid A from *E. coli* published earlier [29], for which an inverted cubic structure was deduced. The diffraction pattern of hexaacyl monophosphoryl lipid A also indicates the existence of a cubic structure, because the relations 5.8 nm $= a_Q/\sqrt{6}$, and 4.14 nm $= a_Q/\sqrt{12}$ hold. The further diffraction maxima could also be indexed by $\sqrt{30}$ and $\sqrt{40}$, but at least the interpretation for the reflection at 2.49 nm is speculative, as for a higher order its sharpness is very unusual. According to previous analyses [6], the broad background in the s range 0.1–0.35/nm indicates the existence of a superimposed unilamellar phase.

A similar complex structural polymorphism is found for the heptaacyl series. Again, the pattern of the bisphosphoryl heptaacyl lipid A is relatively poorly resolved. However, reflections at 5.11 nm, 4.23 nm, and 3.74 nm can be identified which do not correspond to a structure with lamellar periodicity. The aggregate structure of monophosphoryl heptaacyl lipid A can be interpreted to consist of a superposition of a lamellar (reflections at 4.50 nm and 2.25 nm = 4.50 nm/2) and an inverted hexagonal (H_{II}) phase with the reflections obeying the relations 3.05 nm $= 5.25$ nm$/\sqrt{3}$, 2.65 nm $= 5.25/\sqrt{4}$, and 1.95 nm $= 5.25$ nm$/\sqrt{7}$ which is characteristic for the H_{II} structure. The dephosphoryl hexa- and heptaacyl lipids A adopted exclusively lamellar phases with partially two or even more periodicities (lying at 4.39, 3.85, and 2.46 for the hexaacyl sample). The pentaacyl monophosphoryl lipid A exhibited a diffraction peak at 4.72 nm, and two slight shoulders at 7.70 nm and 3.31 nm, and a further peak at 1.92 nm (data not shown). This can be interpreted as resulting from a unilamellar phase with certain non-lamellar characteristics according to interpretations for an enterobacterial lipid A [6].

These data allow the elucidation of the molecular shape. The bisphosphoryl hexaacyl and heptaacyl lipids A adopt a conical molecular shape, i.e., the cross-section of the hydrophobic moiety is larger than that of the hydrophilic part. The respective monophosphoryl lipids A have a nearly cylindrical shape with identical cross-sections of the two moieties. The dephosphoryl lipid A possesses a clear cylindrical shape. The monophosphoryl pentaacyl lipid A is mainly cylindrical, but has a minimal tendency towards a conical shape.

3.6. *Infrared ATR measurements*

The orientational behavior of hydrated lipid A samples, spread on a ZnSe crystal, was investigated with polarized IR light at 26°C. The evaluation of vibrational bands at 1170, 1085, and 1045 cm^{-1} originating from the diglucosamines yielded the results as listed in Table 1. Thus, the hexaacyl and heptaacyl bisphosphoryl lipids A exhibit a strong tilt, in the range 40–50°, of the plane of the diglucosamine backbone with respect to the plane perpendicular to the acyl chain direction, which is significantly lower for the two monophosphoryl lipid A samples.

3.7. *FRET*

The ability to incorporate into a phospholipid matrix corresponding to the composition of the macrophage membrane $PL_{M\Phi}$ mediated by LBP was inves-

Table 1
Infrared dichroic measurements of some hydrated lipids A from *E. carotovora* spread on an ATR ZnSe crystal

Lipid A	Water content (weight%)	Order parameter (S)	Dichroic ratio (R)	Inclination angle (θ)
Monophosphoryl hexaacyl	60	0.92	1.15	39.2 ± 1.0
Bisphosphoryl hexaacyl	58	0.94	1.05	46.0 ± 1.3
Monophosphoryl heptaacyl	23	0.90	1.15	36.5 ± 2.3
Bisphosphoryl heptaacyl	75	0.88	1.03	48.3 ± 1.4

From the measured dichroic ratios of the three diglucosamine vibrational bands at 1170, 1085, and 1045 cm^{-1} and approximations of the order parameter S obtained from the $\nu_s(CH_2)$ at 2850 cm^{-1}, the inclination angle θ between the plane perpendicular to the membrane normal (direction of acyl chains) and the sugar plane was calculated.

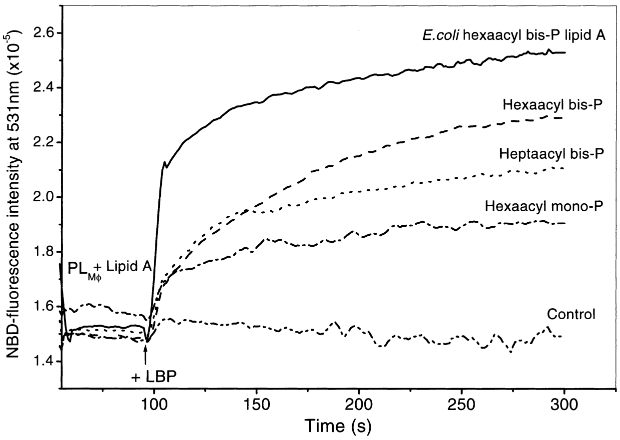

Fig. 6. NBD fluorescence intensity versus time of labeled phospholipid liposomes corresponding to the composition of the macrophage membrane after addition of various lipids A from *E. carotovora* and *E. coli* at $t = 50$ s and subsequent addition of LBP at $t = 100$ s as compared to control buffer.

tigated for some selected lipids A by FRET. The results are given in Fig. 6 for bisphosphoryl hexa- and heptaacyl and monophosphoryl hexaacyl lipid A as compared to bisphosphoryl hexaacyl lipid A from *E. coli* and control buffer (PBS). Clearly, the addition of LBP leads to an immediate intercalation of all lipid A species that is the strongest for the hexaacyl bisphosphoryl species.

3.8. LAL activity

The activity of the different lipids A in the LAL test system as compared to the parent control LPS from *E. carotovora* is given in Table 2. The LPS exhibits some activity down to concentrations of lower than 100 pg/ml, followed by the bisphosphoryl hexaacyl lipid A and the bisphosphoryl heptaacyl lipid A. The response of the monophosporyl lipid A was significantly reduced.

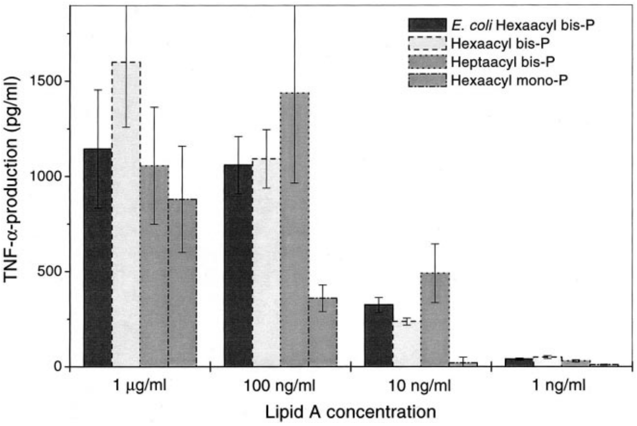

Fig. 7. Production of TNF-α in mononuclear cells induced by bisphosphoryl hexa- and heptaacyl lipid A and monophosphoryl hexaacyl lipid A from *E. carotovora* as compared to bisphosphoryl hexaacyl lipid A from *E. coli*.

3.9. TNF production in mononuclear cells

As measure of biological activity of the various lipids A, their TNF inducing capacity in MNC was determined. The results are presented in Fig. 7. Clearly, within margins of the experimental error the TNF inducing capacity of bisphosphoryl hexaacyl lipid A from *E. coli* and *E. carotovora* is much alike. The heptaacyl lipid A induces significant activity down to a concentration of 10 ng/ml, whereas the monophosphoryl lipid A compound is at least one order of magnitude less active. These results could be confirmed independently for the induction of interleukin-6 (IL-6, data not shown) including also bisphosphoryl pentaacyl and monophosphoryl heptaacyl lipid A. The latter compounds induced

Table 2
Activity of *E. carotovora* LPS and lipid A preparations in the chromogenic *Limulus* test

Concentration (weight/ml) hexaacyl	LPS hexaacyl	Lipid A mono-P[a] heptaacyl	Lipid A bis-P heptaacyl	Lipid A mono-P	Lipid A bis-P
1 µg		+++		+++	
100 ng		++	+++	+++	+++
10 ng	+++	+	+++	++	+++
1 ng	+++	−	++	+	++
100 pg	++		+	−	+
10 pg	−		−		−

[a] Mono-P and bis-P represent monophosphoryl and bisphosphoryl, respectively.

significant IL-6 production at 10 μg/ml and much less at 1 μg/ml (data not shown). It should be noted that a dephosphoryl hexaacyl lipid A preparation was investigated in the biological systems, but did not exhibit any response.

4. Discussion

4.1. General physico-chemical behavior

We have presented a comprehensive physico-chemical analysis of the various lipid A part structures from *E. carotovora* in relation to respective data on the bisphosphoryl hexaacyl lipid A from *E. coli* F515. All lipid A preparations had a high degree of purity and homogeneity. This can be taken from the mass spectrometric analyses (Fig. 2) as well as the monolayer isotherms (Fig. 3), which exhibit a pronounced transition from the liquid expanded into the liquid condensed phase. Therefore, the purity of the samples could be estimated to lie far above 90%, which is comparable only with that of the synthetic lipid A analogues and partial structures. Although *E. coli* lipid A is assumed to be one of the most homogeneous species, it has still a natural heterogeneity, in particular due to the occurrence of pentaacyl and tetraacyl lipid A subspecies [9].

The phase transition experiments indicate clearly an influence of the divalent cation Mg^{2+}, which leads for the bisphosphoryl hexaacyl (Fig. 4B) and heptaacyl (data not shown) lipid A to an increase in accordance to earlier results on lipid A from *S. minnesota* [29] and on Re-LPS [16]. Similar to the bisphosphoryl hexaacyl compound, also for the monophosphoryl hexaacyl lipid A (Fig. 4A) the overall fluidity decreases; however, there is nearly no change in T_c. This can be understood on the basis of the bridging function of divalent cations between neighboring lipid A molecules. This function is strongly hampered by the absence of the 1-phosphate leading to a weaker interaction between adjacent molecules. Concomitantly, this leads to a lower space requirement of the backbone that is detectable from the stronger tendency of monophosphoryl lipid A to adopt lamellar aggregate structures than bisphosphoryl lipid A (Fig. 5).

The remarkable point is the fact that the phase transition of monophosphoryl lipid A from *E. carotovora* is much higher than that of monophosphoryl lipid A from *S. minnesota* [8]. As explanation, there is clear experimental evidence that the HCl treatment used to remove the Kdo units as well as the 1-phosphate also tends to cleave acyl chains (unpublished results), which can be excluded for the chemical treatment used here.

The results for the dephosphoryl lipid A samples, lack of dependence of the phase transition curve on Mg^{2+} and preference for a multilamellar aggregate structure, demonstrate the importance of the phosphate groups for divalent cation bridging between neighboring lipid A molecules as stated above. The preference for lamellar structures and the value of T_c are similar to those found for synthetic hexaacyl dephosphoryl *E. coli* type lipid A (compound No. 503) [28].

The results of the molecular space requirement (Fig. 3) for the bisphosphoryl hexaacyl lipid A, at a lateral pressure of 30 mN m^{-1} of 1.26 nm^2, are in complete accordance with *E. coli* lipid A [30]. The non-linear increase of only 0.09 nm^2 from the hexa- to the heptaacyl compound is an indication of changes in the molecular conformation, probably to a more conical shape. In contrast, the sequence of the molecular areas in the monophosphoryl series indicates nearly linearity. The cross-section of a single acyl chain would be 0.18 nm^2 for the pentaacyl sample, which is known to be the minimum area in the liquid condensed phase [31], 0.19 nm^2 for the hexaacyl compound, and 0.18 nm^2 for the heptaacyl lipid A. Therefore, in the monolayer system no changes of the molecular conformation can be expected. The finding of a very restricted mobility and the weak hydration of the 4′-phosphate within hexaacyl monophosphoryl lipid A from the infrared spectroscopic analysis of the phosphate band $\nu(PO_2^-)$ in the wave number range 1320–1180 cm^{-1} is a clear proof for the fact that (i) the 4′-phosphate is buried in the hydrophobic close region facing probably the interface region (ester, amide) of neighboring lipid A molecules, and (ii) the 1-phosphate may vibrate relatively free in an aqueous environment, i.e., above the membrane plane. This is in excellent agreement with the observed tilt angle of more than 40° for the bisphosphoryl lipid A found from IR-ATR measurements, and is in accordance with results observed for

E. coli type, highly biologically active lipid A [18,32]. An important observation is the occurrence of the inverted hexagonal H_{II} phase in the β phase of the hydrocarbon chains for the heptaacyl monophosphoryl lipid A (Fig. 5B-a). We have found this phase also in the gel phase of a monophosphoryl lipid A from *S. minnesota* R595 LPS (unpublished results), which indicates a high flexibility of the acyl chains in the ordered phase. This can be understood in the context of the reduced acyl chain packing and order in the gel phase being similar to those of monounsaturated phospholipids [16].

4.2. *Biological implications*

We have previously presented the conformational concept of endotoxic activity, which presumes the intercalation of endotoxic molecules into the membrane of immune cells either driven solely by hydrophobic interaction or supported by the action of serum proteins such as LBP [24]. In this membrane, only those lipid A structures, whether alone or as part of the LPS molecule, represent a disturbance at the site of a transmembrane signaling protein which have a conical molecular shape. This leads to initiation of the subsequent biochemical cascade of cytokine induction. Those lipids A adopting a cylindrical shape do not elicit the signaling reaction, but when administered before endotoxically active lipid A, they can block the biological reaction, i.e., they act antagonistically [10].

The basic prerequisite to intercalate into target cell membranes like that corresponding to the composition of the macrophage membrane is fulfilled by all lipid A structures (Fig. 6) investigated in the biological system. Furthermore, our results for the induction of TNF-α in mononuclear cells are in full accordance to the conformational concept. High activity can only be deduced for conically shaped hexa- and heptaacyl lipid A, with the tendency of heptaacyl bisphosphoryl lipid A to be even higher than hexaacyl bisphosphoryl lipid A from *E. carotovora* and *E. coli* (Fig. 7). These lipid A samples have concomitantly a strong inclination of their diglucosamine backbone with respect to the direction of the acyl chains. With increasing tendency to have a more cylindrical shape such as for monophosphoryl preparations also the inclination angle (Table 1) and the biological activities decrease (Fig. 7 and Table 2).

The behavior of hexa- and heptaacyl lipid A from *E. carotovora* is in contrast to the findings for the synthetic hexa- and heptaacyl bisphosphoryl lipid A (compounds 506 and 516). Synthetic heptaacyl lipid A 516 was found to have one order of magnitude lower activity than hexaacyl lipid A 506 [33,34]. It has been proposed that compound 516 has a particular, not completely described molecular conformation differing from that of 506. We could not observe the transition into the H_{II} structure for 516, which most readily takes place for 506 similar to that found for natural lipid A [28]. This would be an indirect indication that 516 is less conical than 506.

The minor tendency of the molecular shape of pentaacyl lipid A to assume a conical conformation may explain the small TNF-α induction of this compound. According to the LAL test (Table 2), there are no clear data with respect to the epitopes within LPS/lipid A. Furthermore, it has been found that endotoxically active as well as inactive LPS/lipid A structures may induce the reaction [35]. Our data suggest that the amount of negative charges is important, since the monophosphoryl compounds exhibited a reduced reaction as compared to the bisphosphoryl compounds irrespective of the acylation degree.

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