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Composition and structure of lipopolysaccharide-human plasma low density lipoprotein complex. Analytical ultracentrifugation, ^{31}P-NMR, ESR and fluorescence spectroscopy studies

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Complexes of *Salmonella thyphimurium* lipopolysaccharide toxin (LPS) with low density lipoproteins (LDL) prepared in vitro have been analyzed. LPS-LDL complexes were found to comprise approx. 0.24 mg LPS/mg LDL protein. The major protein of complexes was apolipoprotein apoB-100 (⩾ 90–95%). Incorporation of LPS molecules into LDL was accompanied by small changes in lipid composition, i.e. the phosphatidylcholine content was diminished by approx. 11% and the free fatty acid concentration was raised 2-fold. Analytical ultracentrifugation showed that insertion of LPS into LDL results in the increase of a portion of particles with higher density (lower flotation coefficient) compared to initial LDL. As was evidenced by ESR, in LPS-LDL complexes, the phospholipid hydrocarbon chains are more ordered than in LDL. ^{31}P-NMR spectra indicated that in LPS-LDL complexes the mobility of phospholipid polar headgroups is restricted in comparison with LDL. Application of the shift reagent (Pr^{3+}) revealed that phospholipid molecules form a monolayer structure on the surface of complexes. Upon binding of LPS to LDL, a maximum of the apoB intrinsic fluorescence was slightly red-shifted (1–2 nm) which may testify that the localization of apoB remains nearly unchanged. For LPS-LDL complexes, the accessibility of apoB fluorophores to quenchers (I^-, Cs^+, acrylamide) did not dramatically differ from that of LDL. It is concluded that rather large amounts of LPS (about 9–10 molecules) can accommodate in one LDL particle without severely perturbing its original composition and structure. Moreover, in the LPS-LDL complexes, oligosaccharide chains of LPS screen notably neither phospholipid polar headgroups nor, what is very important, apoB. LPS-LDL complexes are suggested to be able in vivo to bind to cellular apoB/E receptors, possible LPS receptors and scavenger-receptors of macrophages (monocytes).

Introduction

Lipopolysaccharide toxins (LPS) of both pathogenic and nonpathogenic Gram-negative bacteria circulate in the bloodstream of mammals and humans mainly not in a free state but are associated with serum LP of different density [1–3]. The LPS-LP complexes form through the mediation of some serum proteins, the amount of LPS bound to particular LP being directly proportionate to cholesterol level in the particle [2]. Such complexes are highly stable and can be isolated from plasma or obtained in vitro [2]. The association of LPS with serum LP greatly reduces the cytotoxic effect of LPS as has been found for, e.g., the culture of aorta endothelial cells [4]. However, endotoxin, as a part of the LPS-LP complex, maintains in vivo in the bloodstream much longer (half-life time $\tau_{1/2} \approx 15$ h) than in a free state ($\tau_{1/2} \approx 15$ min), owing to which being able to exert a prolonged action on various types of cells [3]. Besides, quite recently [5] it has been shown for a cellular culture that only one third of all antibodies produced by B-lymphocytes to LPS of *Salmonella typhimurium* is directed against the O-antigenic oligosaccharide chain of molecule, while two thirds are gen-

Abbreviations: LPS, lipopolysaccharide toxin(s); LP, serum lipoprotein(s); LDL, low density LP; HDL, high density LP; KDO, 3-deoxy-D-mannooctulosonic acid; TLC, thin-layer chromatography; FCD, flotation coefficient distribution.

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erated against antigenic determinants of core oligosaccharide and lipid A. If antibodies are yielded to LPS incorporated into an LP particle, the above proportion should be markedly changed.

Earlier it was suggested [2] that LPS of *Escherichia coli* associated with human LDL might initiate (according to the 'response-to-injury' hypothesis [6]) an atherosclerotic process, since LDL (but not HDL) can be retained by the cells of the artery wall. Such an effect may be notably enhanced by hyperlipidemia observed during the infection of animals and humans either with LPS alone [7] or with Gram-negative bacteria [8] which is coupled with the increase in plasma in the amount of β-migrated very low density LP. Also, LPS is known to be able to reduce the activity of LP lipases [7]. Biological bases of LPS-induced hyperlipidemia and its consequences are yet unclear.

The above properties of LPS-LP complexes naturally ought to be related with their composition and structure. Though in several works the binding of LPS to various LP was studied, the complete compositional analysis was reported only for the LPS-HDL complex isolated from rabbit plasma [1]. The more scarce and hypothetical data are available with respect to the structure of LPS-LP complexes. Thus, it was proposed [2] that the LPS molecule binds to the phospholipid monolayer on the surface of LDL via its segment named lipid A [9]. Under that condition, hydrophilic oligosaccharide chains of the core and O-antigenic segments of LPS molecule are assumed to be exposed to the aqueous phase.

In the present work, we have investigated the lipid and protein composition of complexes formed in vitro by LPS of *S. typhimurium* with human LDL in the absence and presence of a fraction of serum proteins. The structure of the obtained complexes was characterized by methods traditionally being used to study the LP structure, namely: ^{31}P-NMR spectroscopy [10,11], acquisition of the intrinsic fluorescence of LDL apolipoprotein in the presence of quenchers [12–15], ESR spectroscopy with the use of spin-labeled fatty acids and analytical ultracentrifugation [16–18].

Materials and Methods

(1) Preparation of complexes of LPS with LDL and determination of their composition. Lipid standards were supplied by Sigma Chemical Co. (U.S.A.). Lipopolysaccharide toxin B of *S. typhimurium* was purchased from Difco Laboratories (U.S.A.). LDL were isolated from the blood of healthy donors by ultracentrifugation as in Ref. 2. Lipids were extracted according to method described in Ref. 19. Complexes of LPS with LDL were prepared as described in Ref. 2; briefly, for a maximal incorporation of LPS into LDL particles during an incubation (5 h, 37 °C), a ratio of 5 mg of LPS per 5 mg of LDL protein was employed. The LPS-LDL$_0$ complex was obtained by a coincubation of the aqueous dispersion of LPS with LDL in the absence of serum proteins. To prepare the LPS-LDL complex, a lipoprotein-deficient fraction ($d > 1.21$) of serum proteins was added into the incubation medium. The complexes obtained were separated from the unbound LPS by ultracentrifugation [2]. The amount of LPS incorporated into the LDL particle was evaluated by two methods: firstly, by determination of total sugar residues content via reaction of a 5% aqueous solution of phenol and concentrated sulfuric acid [20]; and, secondly, by measuring the KDO content [20].

Neutral and phospholipids were determined by TLC using silica gel HPTLC plates supplied by Merck (F.R.G.) and employing the following solvent systems. For neutral lipids: hexane/ethyl ether/acetic acid (15 : 2 : 0.1, v/v) and for phospholipids: methyl acetate/*n*-propanol/chloroform/methanol/0.25% potassium chloride (25 : 25 : 28 : 10 : 7, v/v) [21]. For an application of lipid samples on TLC plates, a special device, Linomat III by Camag (Switzerland), was used. It allows us to administer the exact volume of solution (with an accuracy of up to 0.1 μl) on the very narrow band ($\approx$ 1 mm), which leads to a significant improvement in the achievable separation of components. The measurement of concentrations of lipid components on the plate was performed by quantitative densitometry using a preliminary calibration with various standards and finding a linear region for each component [22]. For this pocedure, a scanning densitometer HPTLC-Scanner (Camag, Switzerland) equipped with a recording integrator SP-4100 (Spectra Physics, U.S.A.) was used. For two series of experiments, phospholipid concentrations were additionally determined by measuring the lipid phosphorus [23]. The results of such an analysis coincided within the 5%-range with those of a quantitative densitometry.

The protein composition of LDL and complexes of LPS with LDL was analyzed by electrophoresis in SDS-polyacrylamide gel slabs with subsequent laser densitometry as described in detail elsewhere [24]. The protein concentration was measured by the method of Lowry et al. [25].

(2) Analytical ultracentrifugation. The polydispersity of preparations of LDL and complexes was studied by a velocity centrifugation in an analytical ultracentrifuge Beckman Model E (U.S.A.) equipped with the UV-scanning optical system and multiplexer. Densitograms were registered with a single channel recorder (Model 110710-10005, Houston Instruments, U.S.A.) adjusted for the accurate measurement of chart speed. The subsequent processing of densitograms was performed with the digitizer Hipad (Houston Instruments, U.S.A.) connected with an Aspect-1000 computer (Bruker, F.R.G.). For a polydispersion analysis, we employed the flota-

tion velocity method to find the differential function of the flotation coefficient distribution $g(S_f)$ (see, for example, Ref. 16). We ascertained that at the rotor speed of 17000 rpm and solvent density (NaBr-solution) of 1.170 g/ml the duration of experiment for LDL should be about 2 h. By this time, the curve of the apparent flotation coefficient distribution (FCD) stopped changing and, hence, it could be taken as the real FCD. The LDL concentration was 0.01% (of the apoprotein content) which corresponded to an optical absorbance of 0.15. Under the above experimental conditions, for a calculation of FCD of LDL samples, it was not necessary to introduce corrections due to the Johnston-Ogston effect and effects of the non-ideality and compressibility of solvent and LDL particles. All experiments were carried out at 20°C, 280 nm wavelength, in the 12 mm double-sector charcoal-filled Epon centerpiece.

(3) ESR spectroscopy. ESR spectra were run in a quartz cuvette (50 μl) at 27°C on a Varian E-109 spectrometer (U.S.A.), fitted with the E-257 variable temperature controller. Two following spin probes were used: 5-doxylstearate (I) obtained from Aldrich (U.S.A.) and 2,2,6,6-tetramethyl-4-octadecyl-oxypiperidinyl-*N*-oxyradical (II) synthesized as described in Ref. 16 and kindly gifted by Dr. A.Yu. Misharin. The incident microwave power was 20 mW (probe I) and 5 mW (probe II); the 100 kHz modulation amplitude was 2 G (probe I) and 0.5 G (probe II). Throughout all ESR experiments we used the Varian E-900 data acquisition system (USA). The mobility of probe I was characterized by the order parameter S calculated as in Ref. 16. For probe II, a rotational correlation time τ was calculated according to Ref. 17. The protein concentration in all samples was 1 mg/ml. The spin probes were used as an ethanol solution at a final concentration in samples of 10^{-4} M. The alcohol content was ≈ 1% (previous experiments [16] showed that a dose of up to 5% v/v of alcohol does not influence on the sedimentation characteristics and ESR spectra of LDL).

(4) ^{31}P-NMR spectroscopy. For NMR assays, preparations of LDL and complexes, after the isolation by ultracentrifugation, were dispersed in a physiological solution (0.9% NaCl) prepared in a mixture of H_2O/D_2O (2:1, v/v). ^{31}P-NMR spectra were obtained on a Bruker WM-250 Fourier-spectrometer (F.R.G.) at 101 MHz frequency in the presence of broadband proton decoupling. For an accurate comparable measurement of integral intensities, gated ^{1}H-decoupling was used. In this case, a sonicated aqueous dispersion of the thion analog of phosphatidylcholine [26] was exploited as the external standard. A protein concentration of LDL and complexes in all samples was 3–5 mg/ml. A $\pi/4$ pulse with an interpulse delay of 3 s was employed. Time-domain spectra were accumulated in 8 K addresses (Aspect-2000 Bruker computer) using a sweep width of 10 kHz. The chemical shift was measured relative to the external standard (85% H_3PO_4).

(5) Acquisition of the intrinsic fluorescence of apoB. Corrected fluorescence spectra (300–500 nm) of apoB in intact LDL and complexes of LPS with LDL were recorded on the Aminco SPF-500 fluorimeter (U.S.A.) with exitation at 280 nm and a bandwidth slit of 5 nm both for the exitation and emission. At this wavelength of exitation, both tryptophan and tyrosine residues (presenting in the apoB-100 molecule at the amount of 37 and 148, respectively [27]) can be excited. However, owing to the reduced sensitivity of tyrosine fluorescence compared with that of tryptophan and considering the noticeable energy transfer form tyrosine to tryptophan residues [13], one may propose that the contribution to the observed fluorescence intensity from tyrosine should be less than that from tryptophan residues [15]. For fluorescence measurements, the aqueous (0.9% NaCl) dispersions of LDL or complexes at minimal protein concentrations (≈ 20 μg/ml) permitting to acquire the high quality spectra were usually taken. Three types of fluorescence quenchers (with the 12% concentration of stock solution for each) were used, namely, I^- and Cs^+ ions, and neutral acrylamide.

Results and Discussion

Composition of complexes of LPS with LDL

As one can readily see from Table I, only a small amount of LPS (approx. 0.07 mg of LPS/mg of LDL protein) spontaneously binds to LDL (the LPS-LDL_0

TABLE I

The content of total carbohydrates and LPS in LDL and complexes of LDL with LPS (mg/mg protein)

Mean values of 4–6 experiments.

Components and methods	LDL	LPS-LDL_0	LPS-LDL
I. Total carbohydrates	0.19 ± 0.02	0.26 ± 0.03	0.40 ± 0.03
II. LPS:			
1. Calculation through determination of total carbohydrates [a]	–	0.08 ± 0.05	0.25 ± 0.05
2. Measurement of KDO content	–	0.06 ± 0.02	0.23 ± 0.04
3. The average value of two above methods	–	0.07 ± 0.05	0.24 ± 0.05

[a] The concentration of LPS was found by subtracting the value of concentration of total carbohydrates in LDL from that in complexes and taking into account that carbohydrate moiety of LPS molecule makes up approx. 85% of its mass [9,30].

complex), whereas the protein stimulated transfer of LPS to LDL results in the incorporation of about 0.24 mg of LPS/mg of LDL protein (the LPS-LDL complex). It is interesting from these weight ratios of LPS/apolipoprotein to try to infer the molar ones. As will be shown below, the main apolipoprotein of the complexes obtained, as well as of intact LDL[28,29], is apolipoprotein B-100 (apoB-100). The molecular mass of apoB-100 equals to 513 kDa according to a recent report [27]. However, the molecular mass of LPS of *S. typhimurium* can hardly be exactly evaluated, because the composition of the O-antigenic oligosaccharide chain of the LPS molecule for different strains varies significantly [9]. Nevertheless, taking into account that the average number of repeating units of the O-chain equals to approx. 8 [30], we suppose that the molecular mass of about 13 kDa might be used for an approximate estimate. Then, for the LPS-LDL complex, we obtain that about 9–10 moles of LPS bind per 1 mole of apoB-100. Or, remembering that the LDL particle contains one molecule of apoB-100 [28], we derive that, in case of the LPS-LDL complex, on the average, 9–10 molecules of LPS are associated with one LDL particle. For the LPS-LDL_0 complex, such a mean value is three molecules of LPS per LDL particle. Since LPS-LDL complexes, which were similar with regard to LPS content, were obtained for LPS of *E. coli* both in vitro and in vivo experiments [2], it is reasonable to suggest that antibodies to LPS probably do not play any important role in the complex formation process. Perhaps, some components of the complement system may perform functions of the LPS carrier, e.g., a C_3 component capable of binding directly to LPS [3,31].

The data of Table II demonstrate that the lipid composition of complexes obtained does not dramatically differ from that of the initial LDL, which resembles the earlier reported results [29,32]. Though, it may be noted that as the amount of LPS incorporated increases, the content of phosphatidylcholine (and total phospholipids) in the complex somewhat diminishes. A change in the composition of neutral lipids caused by incorporation of LPS into LDL manifests itself mainly by the pronounced rise in free fatty acid content (Table II). The observed decrease in phosphatidylcholine content in complexes could, in our opinion, testify that LPS molecules, being inserted in the surface lipid monolayer of LDL particle by their hydrocarbon chains of lipid A (by analogy with the LPS interaction with a lipid bilayer of biological membranes [33]), are able to eject from it some phosphatidylcholine molecules. The elevation of free fatty acid level in complexes may have physiological consequences, since recently it was found that higher concentrations of these substances stimulate the LP catabolism [34].

An analysis of protein components of LDL, LPS-LDL_0 and LPS-LDL complexes showed that for all three preparations the protein composition is approximately (within the experimental error) identical, namely, apoB-100 makes up more than 90–95% of all proteins (electrophoretogram not presented). In some experiments, the presence of other minor components was observed: the apoB-100 fragments of high molecular mass (3–5%); apoE and apoC (≈ 3% altogether); albumin (≈ 3%). It is noteworthy that serum proteins taking part in the LPS binding to LDL do not possibly associate with complexes at the detectable quantities.

TABLE II

The composition of LDL, LPS-LDL_0 and LPS-LDL complexes

Mean values of 4–6 experiments. The LPS preparation contained ⩽ 5% (w/w) of phospholipids, the bulk of which was presented by phosphatidylethanolamine (60%) and phosphatidylglycerol (23%) with minor quantities of cardiolipin (5%) and the lyso-form of these phospholipids; ⩽ 2% (w/w) of neutral lipids among which free fatty acids dominated; ⩽ 3–5% (w/w) of total protein admixtures.

Components	LDL	LPS-LDL_0	LPS-LDL
I. General composition of particles (weight %)			
1. Phospholipids	23.0 ± 1.0	22.0 ± 1.2	20.5 ± 1.2
2. Protein	19.7 ± 1.0	20.0 ± 1.0	20.0 ± 1.1
3. Total neutral lipids:	53.5 ± 5.6	53.0 ± 5.5	51.0 ± 5.3
4. Carbohydrates	3.8 ± 0.4	5.2 ± 0.5	8.3 ± 0.7
II. Phospholipid composition (weight %)			
1. Lysophosphatidylcholine	0.9 ± 0.3	1.6 ± 0.5	1.5 ± 0.4
2. Sphingomyelin	21.9 ± 1.6	24.3 ± 1.9	25.9 ± 1.7
3. Phosphatidylcholine	68.2 ± 2.5	63.9 ± 3.3	60.9 ± 2.3
4. Phosphatidylserine	0.8 ± 0.4	1.0 ± 0.4	1.2 ± 0.4
5. Phosphatidylinositol	2.6 ± 0.4	1.3 ± 0.5	2.7 ± 0.4
6. Phosphatidylethanolamine	5.4 ± 0.4	6.0 ± 0.6	6.4 ± 0.6
III. Composition of neutral lipids (weight %)			
1. Free cholesterol	17.0 ± 1.8	16.8 ± 2.2	19.0 ± 2.0
2. Free fatty acids	3.0 ± 0.8	5.4 ± 1.6	6.2 ± 2.1
3. Triacylglycerol	16.0 ± 2.7	16.7 ± 2.9	17.0 ± 2.6
4. Esterified cholesterol	57.2 ± 5.3	53.0 ± 5.6	51.3 ± 5.2

Structure of complexes of LPS with LDL

(1) Analytical ultracentrifugation. Fig. 1 demonstrates that the flotation coefficient distributions for initial LDL and the LPS-LDL_0 complex are practically undistinguishable. On the contrary, the distribution curve for the LPS-LDL complex clearly shows that, in this case, the portion of particles with higher flotation coefficients diminishes and the portion of particles with higher density (lower flotation coefficient, S_f) increases. These findings correlate well with results of chemical composi-

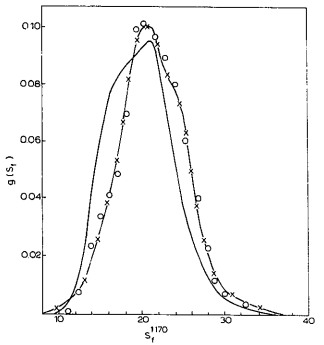

Fig. 1. Profiles of the flotation coefficient distribution $g(S_f)$ for: ×------×, intact LDL; o-----o, LPS-LDL$_0$ complex; ———, LPS-LDL complex. On the abscissa axis the $S_f^{1.170}$ values are presented (Svedberg units). For details see text.

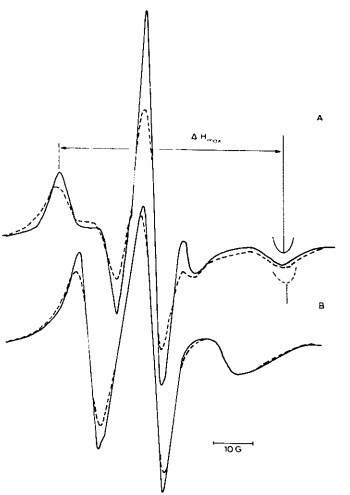

Fig. 2. ESR spectra of LDL (———) and the LPS-LDL complex (- - -): A, probe I; B, probe II. For experimental details see text.

tion (Table II), i.e., the LPS-LDL complex, in comparison with LDL and with the LPS-LDL$_0$ complex, contains a larger portion of carbohydrates and a smaller portion of lipids.

(2) ESR spectroscopy. The packing of hydrocarbon chains of phospholipid molecules in the surface monolayer of LDL and complexes was analyzed with the aid of 5-doxylstearate as a hydrophobic probe by calculating the order parameter, S, from ESR spectra (Fig. 2A) as described in detail in Ref. 18. Data of Table III evidence that the most ordered arrangement of phospholipid fatty acide chains in the proximity of parmagnetic fragment of 5-doxylstearate is observed for the LPS-LDL complex (note especially the difference in ΔH_{max} values). The LPS-LDL$_0$ complex and LDL are characterized by nearly equal and significantly lower order parameters. Some authors (for a review, see Ref. 9) have reported that insertion of LPS into biological or model phospholipid bilayers results in the tougher packing of phospholipid acyl chains and 10-fold decrease in the rate of lateral diffusion of molecules in the membrane. A somewhat more ordered organization of the hydrophobic interior of the surface monolayer in the LPS-LDL complex (containing the highest amount of toxin) might ensure from such a 'structurizing' action of LPS. The dynamic properties of phospholipids were assessed with a probe II, the paramagnetic fragment of which is localized in the monolayer at the level of phosphate groups of phospholipid molecules. Hence, one may think that the behavior of paramagnetic probe II should reflect the influence of neighboring phosphate groups. The tougher the arrangement of phospholipid headgroups is, the more restricted motion of probe II would be observed (assuming it is randomly distributed in the phospholipid monolayer). The ESR spectrum of probe II, in contrast to that of a probe I (Fig. 2B), reveals nearly isotropic characteristics what points out to the only slightly hindered mobility of paramagnetic group. In case of probe II, the rotational correlation time (τ) values were calculated (Table III). For the LPS-LDL complex, the significantly longer τ value

TABLE III

Parameters derived from ESR spectra (Fig. 2) characterizing the behavior of paramagnetic probes in LDL and complexes of LPS with LDL

Mean values of 3–4 experiments.

Sample	Probe I		Probe II
	ΔH_{max} (G)	order parameter, S	rotational correlation time τ (ns)
1. LDL	58.0 ± 0.2	0.693 ± 0.008	4.80 ± 0.21
2. LPS-LDL$_0$	58.0 ± 0.2	0.695 ± 0.009	4.57 ± 0.25
3. LPS-LDL	59.5 ± 0.5	0.716 ± 0.010	5.34 ± 0.26

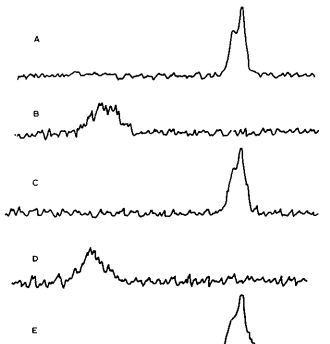

Fig. 3. ^{31}P-NMR spectra of intact LDL (A); LDL + Pr^{3+} (B); LPS-LDL_0 complex (C); LPS-LDL_0 complex + Pr^{3+} (D); LPS-LDL complex (E); LPS-LDL complex + Pr^{3+} (F).

compared with that for LDL and the LPS-LDL_0 complex (which are practically the same) was found. This fact points out to the more hindered mobility of paramagnetic fragment which might be the consequence of some additional constraints imposed by phospholipid headgroups in the presence of LPS. Summing up the ESR findings, we can conclude that incorporation of LPS into LDL in large enough quantities leads to a somewhat thougher molecular organization of nonpolar regions of the surface lipid monolayer and restricted mobility of molecular segments near the water/lipid interface.

(3) ^{31}P-NMR spectroscopy. The additional important information about the structure formed by phospholipid molecules on the surface of complexes can be drawn out of ^{31}P-NMR spectra. The aqueous dispersions of LDL produce the ^{31}P-NMR spectra of fairly high resolution (see Fig. 3A, and also Refs. 10 and 11). Two semi-resolved narrow signals from two dominant phospholipids in LDL are usually observed: the upfield one is due to phosphatidylcholine molecules, and the downfield one is attributable to sphingomyelin molecules; the intensities of the signals being proportional to their concentrations in LDL. Comparison of the intensity of the total phospholipid signal with the external standard has shown that approx. 80% of all phospholipid molecules contribute to the narrow signals. Close results were procured earlier both with ^{1}H-NMR [35] and with ^{31}P-NMR spectroscopies [10,11] when it was found that the portion of strongly immobilized polar headgroups (signals from which are broadened towards the baseline) of phospholipids in LDL makes up 21–28%. The total halfwidth (linewidth at half-height), $\Delta\nu_{1/2}$, of the signals from phosphatidylcholine and sphingomyelin in the spectrum of intact LDL equals to approx. 99 ± 2 Hz (Fig. 3A). The addition into the aqueous medium of a hydrophilic shift reagent (6 mM Pr^{3+}) causes the downfield movement of all ^{31}P-NMR signal (up to 10 ppm) what points out to the monolayer organization of phospholipid molecules on the surface of LDL (see Fig. 3B, and Ref. 11).

In the ^{31}P-NMR spectrum of LPS-LDL_0 complex (comprising only a small amount of toxin), in the absence of Pr^{3+} ions (Fig. 3C), one can remark that total halfwidth of phospholipid signals ('phosphatidylcholine + sphingomyelin') slightly increases $\Delta\nu_{1/2} \approx 104 \pm 2$ Hz, and separation of the phosphatidylcholine and the sphingomyelin signals becomes more difficult (signals arising from LPS phosphorus nuclei are not observed due to the relatively low phosphorus content [9,30]) The intensity of the total phospholipid signal measured in relation to the external standard corresponds to 75–80% of all phospholipids present. After Pr^{3+} ions have been administered, only one broad signal shifted downfield, as for LDL, is observed (Fig. 3D). This fact evidences that in the LPS-LDL_0 complex the overwhelming majority of phospholipid phosphate groups remain accessible to paramagnetic cations. The magnitude of Pr^{3+}-induced shift of phosphorus magnetic resonance [36,37], $\Delta\delta = \delta_{Pr} - \delta_0$, in this case, is 11 ppm which differs insignificantly from that of control.

For the LPS-LDL complex containing the highest concentration of LPS, in the ^{31}P-NMR spectra there are much more pronounced changes. Thus, in the absence of shift reagent (Fig. 3E), the halfwidth of the total phospholipid signal increases up to 111 ± 3 Hz and distinct signals from phosphatidylcholine and sphingomyelin can hardly be resolved. A calculation shows that, in this case, no more than 60–70% of all phospholipid molecules produce the relatively narrow signals. Hence, the presence of LPS somewhat increases the share of highly immobile polar moieties of phospholipid molecules. The application of Pr^{3+} ions induces a lowfield shift of the entire phospholipid signal (Fig. 3F), i.e., again the bulk of phospholipid headgroups is not screened from a hydrophilic probe. For the LPS-LDL complex, the magnitude of the induced shift is maximal (approx. 13–15 ppm) which is probably due to the presence of several negatively charged groups in the LPS molecule. The augmentation of halfwidth of phospholipid signals observed upon binding of LPS to LDL

TABLE IV

Quenching of the intrinsic fluorescence of apoB in LDL and complexes of LPS with LDL

Mean values of 4–6 experiments.

Sample	Without quencher		Maximum percentage of quenching [b]		
	λ_{max} [a] (nm)	Relative intensity of fluorescence	KI	CsCl	Acrylamide
1. LDL	326 ± 0.4	1.00	55 ± 4	44 ± 3	58 ± 6
2. LPS-LDL_0	328 ± 0.5	0.92 ± 0.06	63 ± 5	52 ± 4	75 ± 7
3. LPS-LDL	327 ± 0.5	1.07 ± 0.08	50 ± 4	55 ± 4	67 ± 6

[a] After addition of quenchers λ_{max} was slightly blue-shifted (0.5–1.0 nm) and peak halfwidth was increased by 2–4 nm.
[b] Values were calculated from plots of Fig. 4.

may be the consequence of both the decreased mobility of the phospholipid head groups on the surface of the particle, and the lowered rate of tumbling of the particle, as a whole (owing to lengthy oligosaccharide chains of the inserted LPS molecules).

It is reasonable to propose that incorporation of LPS in the LDL particle, increasing the density of lipid packing in the surface monolayer, can cause the expulsion to the outer medium of phosphatidylcholine molecules which have the most spacious head groups of all phospholipids. Conversely, phosphatidylethanolamine molecules, being 'akin' to LPS [9], can ease the accommodation of toxin in a lipid monolayer. On the surface of the LDL particle, between the LPS acyl chains there might be likely rather roomy cavities which can be advantageously filled by cholesterol molecules. This can account for the fact that LPS preferably binds to those LP where cholesterol content is higher [2].

(4) Fluorescence spectroscopy. The possible alterations in the accommodation of apolipoprotein on the surface of LDL after the LPS incorporation were approximately estimated by acquisition of the intrinsic fluorescence of apoB-100. As it can be seen from data of Table IV, the binding of LPS to ' : LDL particle induces a small red-shift of the maximum of the apoB fluorescence compared with intact LDL, for the LPS-LDL_0 complex this shift being slightly larger (2 nm) than for the LPS-LDL complex (1 nm). These differences, though very small ones, might give a hint of the probability that the incorporation of LPS is accompanied by minor changes (if any) in the localization of apoB molecule under which its certain fragments, on the average, become exposed to a somewhat more hydrophilic environment than in LDL.

More detailed information on whether such putative rearrangements of apoB did occur upon binding of LPS

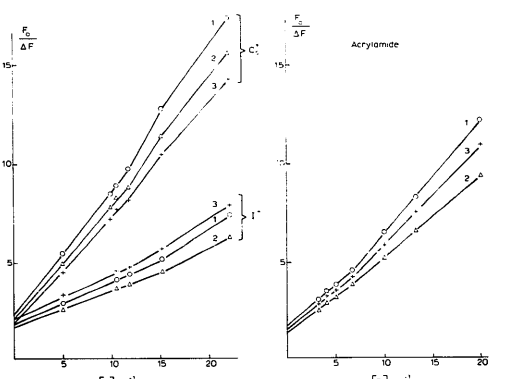

Fig. 4. Stern-Volmer plots (modified equation) for intact LDL (1); LPS-LDL_0 complex (2); LPS-LDL complex (3).

to LDL could be obtained with the help of fluorescence quenchers of different charge and size. Earlier it was ascertained [12,14,15] that the accessibility of apoB fluorophores to quenchers and, hence, the localization of apoB in LDL can be characterized using a modified Stern-Volmer equation:

$$F_0/(F_0 - F) = 1/([Q]f_a K_Q) + 1/f_a$$

where F_0 is the initial fluorescence intensity; F is the fluorescence intensity in the presence of quencher; [Q] is the quencher concentration; f_a is the 'effective' fractional maximum fluorescence accessible to the quencher; K_Q is the 'effective' quenching constant.

Extrapolating experimental plots of $F_0/\Delta F$ vs. $1/[Q]$ (Fig. 4) under the condition of $1/[Q] \to 0$, it is possible to derive the f_a value as 1/intercept (Table IV). As one can readily see, these dependences, taken as a whole, are substantially non-linear in all cases which probably points out to the significant heterogeneity of quenching rates for different tryptophan (and tyrosine) residues of apoB in LDL and complexes. A similar nonlinearity of the Stern-Volmer dependence for LDL with the use of I^- ions was noted earlier [15]. Though this circumstance does not allow to determine total K_Q constants, the values of f_a can be obtained from dependencies of Fig. 4. For LDL, data of Table IV show that about 50–60% of the intrinsic fluorescence of apoB can be quenched which testifies that a substantial part of the apoB molecules is located near the water/lipid interface. Earlier some authors have reported [15] that no more than 20% of the apoB fluorescence in LDL_2 is observed upon addition of I^- ions, while others have concluded [12] that I^- ions quench only 40% of that. We suppose that this discrepancy can be at least partly explained by the different lipid composition of the LDL studied.

For the LPS-LDL complexes, approximately the same portion (as for LDL) of the apoB fluorescence disappeared in the presence of quenchers (Table IV), i.e., the accessibility of the apoB fluorophores to each quencher, on the average, remained approximately unchanged. Moreover, the effectiveness of quenchers did not significantly depend on their charge and size. From these findings, two principal conclusions could be drawn. Firstly, the embeddedness of the apoB molecule, as a whole, into the surface lipid monolayer is not markedly altered upon binding of LPS to LDL. However, the occurrence of minor changes in the locations/orientations of some of the numerous fluorescing residues of apoB can not be, certainly, ruled out. Or, in other words, basing on such an approach, it is practically impossible to detect subtle changes in the environment of receptor sites in apoB (see Ref. 38, and references therein). Secondly, keeping in mind that the presence of LPS does not reduce the access of quenchers (particularly of acrylamide) to apoB fluorophores, it is reasonable to propose that the lengthy oligosaccharide chains of LPS do not notably screen the surface of apoB and, hence, its receptor sites.

Summing up all the results presented here, one can make the following general remarks. The incorporation of LPS molecules into the LDL particle affects dramatically neither its composition nor its structure. As for natural LDL, the hydrophobic core of complexes is still encircled by the surface monolayer formed by phospholipid molecules; apoB-100 is practically the sole protein present. Acyl chains of the bound LPS molecules do penetrate in a lipid surface layer, because an ordering effect has been revealed for both polar and nonpolar segments of molecules positioned there. Oligosaccharide chains of LPS directed to the aqueous phase are oriented in such a manner that they shield detectably neither phospholipid polar headgroups nor, what is utterly important, surface segments of apoB. This enables us to suggest that in vivo the LPS-LDL complex may specificially interact with the cellular apoB/E receptors which in vitro has been already demonstrated for monocytes [2]. On the other hand, the loose O-antigenic oligosaccharide chains of the incorporated LPS can provide for complexes an opportunity to bind to the possible cellular receptors of LPS [5,39,40]. Besides, it is now well known (e.g., see Ref. 2 and references therein) that modification of the LP surface by some agents leads to the perturbation of LP catabolism shifting it to the binding to scavenger-receptors of macrophages (monocytes) as a result of which the latters accumulate the substantial amounts of cholesteryl esters. This can initiate the formation of foam cells and the development of atherosclerotic lesions [41]. The incorporation of LPS into LDL was shown to influence the activity of scavenger-receptors of monocytes [2]. It appears reasonable to propose that the association of LPS (and some other toxins [42] and viruses [43]) with plasma LP promoting their subsequent interaction with scavenger-receptors of macrophages may be one of the main ways to neutralize and remove toxic agents from the organism. Finally, the fact that it is lipid A (through which toxin binds to LDL) that represents the most 'pathogenic' fragment of LPS molecule [9] could account possibly for the reported lowered cytotoxicity of LPS forming a stable complex with LDL [4]. It is only logically to suggest that the inactivation of other toxins via association with plasma LP results from neutralizing of particular fragments of their molecules which are responsible for the incorporation of toxin in the lipid bilayer of the cell membrane.

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