Lipoproteins in Innate Immunity
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Citation for published version (APA):
Levels, J. H. M. (2002). Lipoproteins in innate immunity

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Chapter II

CHAPTER II

Distribution and kinetics of lipoprotein-bound endotoxin

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(Infection and Immunity, volume 68, No.5, 2001.)
Lipopolysaccharide (LPS), the major glycolipid component of Gram-negative bacterial outer-membranes, is a potent endotoxin responsible for pathophysiological symptoms characteristic of infection. The observations that the majority of LPS is found in association with plasma lipoproteins has prompted the suggestion that sequestering of LPS by lipid particles may form an integral part of a humoral detoxification mechanism. Previous studies on the biological properties of isolated lipoproteins have employed differential ultracentrifugation for the separation of the major subclasses. To preserve the integrity of the lipoproteins we have analyzed the LPS distribution, specificity, binding capacity and kinetics of binding to lipoproteins in human whole blood or plasma using high-performance gel-permeation chromatography (HPGC) and fluorescent LPS of three different chemotypes. The average distribution of O111:B4, J5 or Re595 LPS in whole blood from 10 human volunteers was: HDL 60% (±8%), LDL 25% (±7%) and VLDL 12% (±5%). The saturation capacity of lipoproteins for all three LPS chemotypes was in excess of 200 μg/ml. Kinetic analysis however, revealed a strict chemotype-dependence. The binding of Re595 or J5 LPS was essentially complete within 10 min and subsequent redistribution among the lipoprotein subclasses occurred to attain similar distributions as O111:B4 LPS at 40 min. We conclude that under simulated physiological conditions, the binding of LPS to lipoproteins is highly specific. HDL has the highest binding capacity for LPS, the saturation capacity of lipoproteins for endotoxin far exceeds the LPS concentrations measured in clinical situations and that the kinetics of LPS association with lipoproteins display chemotype-dependent differences.
**Introduction**

The lipopolysaccharide (LPS) components of Gram-negative bacterial outer membranes are potent endotoxins responsible for hemodynamic, hematological and metabolic changes observed during severe infection. Activation of responsive cells of the host immune system by low concentrations of LPS, results in the production of high levels of endogenous mediators of inflammation such as tumor necrosis factor alpha (TNF-α and interleukines, (IL-1β, IL-6 and IL-8), which are capable of sustaining the inflammatory state. Among the observed metabolic changes in patients, are profound disturbances in plasma lipid profiles (1), (2), (3) that may also be induced in experimental animals by LPS challenge (4). Lipid metabolism appears to be extensively regulated during the host response to infection, however, increased cytokine levels do not solely appear to be responsible for the characteristic alterations in plasma lipid profiles. It has recently been suggested that disturbances in lipid metabolism may, in fact form part of the host defense because the immune response is tightly linked to the metabolic response (5). Lipopolysaccharide binding protein (LBP) is an acute phase protein (6) that plays a central role in the attenuation of the cellular response to endotoxin by the presentation of LPS monomers to membrane-bound CD14 (mCD14), on monocytes and macrophages (7), (8). On the basis of structural homology with bactericidal permeability/increasing protein (BPI) located in the granulocytes (9), LBP together with cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP), have recently been described as belonging to a family of lipid transport proteins (10). CETP and PLTP are known to be associated with high-density lipoprotein (HDL) particles and to be essential for lipoprotein remodeling (11). Recent evidence suggests that LBP like other members of the lipid transport family is bound to HDL (12), (13). A number of studies have demonstrated that LPS binds to all of the lipoprotein classes (14), (15), (16) and inhibits activity of proteins essential for lipoprotein homeostasis, such as CETP, PLTP as well as lecithin cholesterol acyltransferase (LCAT) and lipoprotein lipase (5). The mechanism of transfer of LPS to reconstituted HDL (R-HDL) particles in vitro, has only recently been elucidated and was shown to require LBP and soluble CD14 (sCD14) (17). The rate of LPS transfer to native lipoproteins in whole plasma appears to occur at a similar rate, but shows no sCD14-dependence (17).
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Several observations indicate that in humans lipid transport and LPS-detoxifying mechanisms converge on similar routes. Lipoprotein-bound LPS has been shown to be less biologically active \textit{in vitro} (18), (19), (20), (16). Generally, clearing of LPS from plasma is enhanced when LPS is associated with lipoproteins and results in increased biliary excretion (21). The rate of hepatic clearing of LPS appears to be chemotype dependent. LPS from a bacterial strain with a rough phenotype contains shorter O-antigenic polysaccharide chains, is cleared more rapidly than LPS from a smooth strain (22). Although these observations suggest that lipoproteins constitute an endogenous LPS-detoxification system (23), the mechanism and dynamics of the association of different LPS chemotypes with native lipoproteins, as well as the biologic consequences of these interactions remain to be elucidated. The majority of LPS binding studies (20), (24), (15), (25) have employed differential ultracentrifugation for the isolation of lipoproteins and analysis of lipoprotein-bound LPS. However, a number of studies have indicated that shear-forces generated during high-speed centrifugation of, results in extensive stripping of protein components of the lipoprotein particles (26), (27), (28), (29), (30). In this context, the relatively gentle lipoprotein separation technique of size-exclusion chromatography has been described as eminently more suitable for maintenance of the compositional integrity of lipoproteins and associated proteins that constitute native particles (31), (32), (33), (34). Here we present the LPS binding characteristics of the major lipoprotein classes determined with the use of high performance gel-permeation chromatography (HPGC) and three different chemotypes of fluorescently labeled LPS. We report a comparison of the lipoprotein distribution, binding capacity and kinetics of smooth \textit{E. coli} serotype O111:B4, the rough \textit{E. coli} serotype J5 and the deep rough \textit{Salmonella typhimurium} Re595 LPS under simulated physiological conditions.

Materials and Methods

Reagents and materials

Lipopolysaccharides of the highest purity were obtained from commercial sources. 
\textit{Escherichia coli} O111:B4 LPS was from Sigma Chemical Co. (St Louis, MS), \textit{E. coli} J5 (Re) and \textit{Salmonella typhimurium} Re595 LPS were from List Biological Laboratories (Campbell, CA) or Calbiochem-Novabiochem Intl. (La Jolla, CA). Pyrogen-free distilled water used throughout the experiments, was from Ecotainer (Braun Medical AG, Melsungen, Germany). The fluorescent
Lipoprotein associated LPS

labels NBD-F and BODIPY-R6G were obtained from Molecular Probes Inc. (Eugene, OR). PAP reagent for post-column cholesterol detection was from Bio-Merieux (Marcy l’Etoile, France). Pyrogen-free heparin was purchased from LEO Pharmaceuticals B.V. (Weesp, Netherlands) and Tris-hydroxymethylaminomethane, from Boehringer Mannheim (Mannheim, Germany). NaCl, Tween-20, di-isopropyl ether (DIPE) and n-butanol of the highest purity were purchased from Merck, (Darmstadt, Germany). TNF-α was determined with the Pelikine human TNF-α ELISA analysis kit (CLB, the Netherlands). Centricon-100 filters were from Amicon (Beverly, MA). apo B and apo A-1 levels of lipoprotein fractions were measured by an automated turbidometric assay using the APA and APB kit on an Array Protein System Nephelometer (Beckman, Mijdrecht, Netherlands). NaIO₄ and Purpald reagent for LPS quantification was obtained from Aldrich Sigma (Steinheim, Germany)

Methods

BODIPY labeling of O111 LPS

Purified E. coli O111:B4 LPS was labeled using the BODIPY-R6G oligonucleotide amine labeling kit (Molecular Probes, Eugene, OR) by modifications of the manufacturer’s protocol for oligosaccharide labeling. LPS was prepared for labeling by sonication of a suspension at a concentration of 2 mg/ml in pyrogen-free water with a Branson sonifier at maximum output for a total of 10 min on ice. Using a molecular weight of 12 kDa for the average size of the O111:B4 monomer (35, 36) LPS at a final concentration of 1 mg/ml in 0.1 M sodium bicarbonate buffer pH 8.3, was derivatized in polypropylene tubes by addition of a 5-fold molar excess of BODIPY-R6G dissolved in DMSO and the reaction allowed to proceed for 2 h in the dark at room temperature. Non-conjugated BODIPY label remaining after the derivatization was allowed to react with a 20-fold molar excess of glycine for a further 30 min. The BODIPY-LPS conjugate was separated from BODIPY-glycine by gel-filtration on a 5 ml Sephadex G15 column (Pharmacia Biotech, Inc. Sweden) using pyrogen-free water. The BODIPY-LPS micelles elute in the void volume, while BODIPY-glycine is retained by the matrix. The efficiency of label incorporation was determined by measurement of the optical density (O.D) at 528 nm using the quoted extinction coefficient of 70,000 cm⁻¹ M⁻¹ and the stoichiometry of labeling was found to be approximately 1 BODIPY : 1 LPS. Labeling of O111:B4 with NBD resulted in considerably lower labeling efficiencies. The concentration of the peak fraction of labeled LPS determined by the KDO assay (37) was 0.76 mg/ml.

NBD labeling of J5 and Re595 LPS

Purified J5 or Re595 LPS were labeled using NBD-F (Molecular Probes, Eugene, OR) according to modifications of the manufacturer’s instructions for oligosaccharides. LPS was
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prepared for labeling by sonication of J5 LPS for a total of 5 min and Re595 LPS for 2.5 min as described for O111:B4. Using molecular weights of 3800 kDa for J5 and 2300 kDa for Re595, for the average size of the monomers, LPS suspensions at 1 mg/ml in 0.1 M sodium bicarbonate buffer pH 9.0, were labeled by addition of a 5-fold molar excess of NBD-F dissolved in DMF and the reaction allowed to proceed in the dark for 1 h at room temperature. Non-conjugated NBD label remaining after the derivatization was allowed to react with a 20-fold molar excess of glycine for a further 30 min. Derivatized NBD-LPS was separated by gel-filtration on Sephadex G15 as described before. The efficiency of labeling was determined by measurement of the O.D at 465 nm using the quoted extinction coefficient of 8000 cm$^{-1}$ M$^{-1}$ and the stoichiometry of labeling found to be approximately 1 NBD:4 J5-LPS and 1 NBD:6.5 Re595-LPS. Labeled LPS was monomerized by heating for 5 min at 100 °C in the presence of 2% (w/v) SDS and characterized by RP-HPLC on a C$_{18}$ column using 20% (v/v) ethanol containing 0.5% (w/v) SDS as eluent. No free label was detected in any of the LPS preparations. BODIPY O111:B4 eluted in four major peaks, which is consistent with the known heterogeneity of O111:B4 (38). By contrast single peaks were evident for the homogeneous J5 and Re595 chemotypes. The concentration of the peak fractions of J5 and Re595 LPS were 0.73 and 0.83 μg/ml, respectively determined by KDO assay. LPS suspensions could be stored for up to 6 months at 4 °C without appreciable loss of fluorescence yield. Derivatization of J5 or Re595 LPS with BODIPY in our hands, yielded significantly lower labeling-efficiencies compared with the NBD fluorophore. Additionally, BODIPY or FITC derivatives of Re595 LPS have previously been described as having variable biological activities (39). We therefore chose to minimize the potential influence of the label on the biophysical and/or biological properties of the smaller LPS preparations (J5 and Re595) by using a fluorescent label of lower molecular weight (NBD) for these chemotypes.

Biophysical-chemical properties of fluorescent LPS

To determine whether the fluorescent label influences the biophysical-chemical behavior of derivatized LPS, competition analysis was done using samples containing mixtures of labeled and unlabeled LPS of the same chemotype, in defined ratios from 6.25% (w/w) labeled LPS :93.75% (w/w) unlabeled LPS to 100% (w/w) labeled LPS to achieve final concentrations of 200, 300, and 200 μg/ml plasma, which approach saturation for O111:B4. J5 and Re595 LPS, respectively. The volume of the LPS mixtures added to aliquots of plasma was kept constant and incubations were for 20 min at 37 °C.

Blood sampling and handling

Whole blood was drawn from healthy volunteers after informed consent, by venipuncture and generally collected in pyrogen-free polypropylene tubes containing heparin (2 U/ml) or in some instances tubes containing sodium citrate (Becton and Dickinson, Lincoln Park, NJ). Blood or
Lipoprotein associated LPS

plasma obtained by centrifugation (1000 x g for 20 min at 12 °C) was always used in the experiments within 1 h after collection.

**Plasma delipidation**

Total delipidation of plasma was essentially according to Cham and Knowles (40). Aliquots of 2.5 ml of fresh citrated plasma were added to 5 ml n-butanol/di-isopropyl ether (DPE) 40:60 (v/v) in 14 ml polypropylene tubes (Becton & Dickinson). The mixture was gently agitated on a roller for 30 min. at room temperature and centrifuged at 1000 x g for 10 min. at room temperature for phase separation. The aqueous phase was collected by needle puncture at bottom of the tubes and re-extracted with 2 volumes of DPE for 2 minutes. Residual butanol was removed under vacuum at 37 °C for 5 minutes followed by a continuous stream of nitrogen. Delipidated plasma was generally used immediately for further experimentation.

**Separation of the major lipoprotein classes by HPGC**

The system contained a PU-980 ternary pump with and LG-980-02 linear degasser, a FP-920 fluorescence and UV-975 UV/VIS detector (Jasco, Japan). An extra P-50 pump (Pharmacia Biotech, Sweden) was used for on-line cholesterol detection. The separation matrix was Superose 6 HR 10/30 (Pharmacia Biotech, Sweden). The injection volume was 60 µl of plasma diluted 1:1 with TBS, pH 7.4 (30) containing 0.005 % (v/v) Tween-20, pH 7.4. (TBST) and development of the chromatograms was with TBST at a continuous flow rate of 0.31 ml/min. Computer analysis of the chromatograms were with Borwin Chromatographic software, version 1.23 (JMBS Developments, Le Fontanil, France).

**Distribution, lipoprotein-binding capacity and kinetics of different LPS chemotypes**

For the LPS distribution experiments 50 - 1 aliquots of labeled LPS in saline were added to 0.5 ml portions of fresh whole blood in polypropylene tubes for a concentration of 20 - 30 µg/ml and incubated for 1 hour at 37 °C. For the saturation experiments, LPS was used at concentrations in excess of 200 µg/ml. Chromatographic profiles of the association of fluorescent LPS with lipoproteins in plasma samples were analyzed in plasma by HPGC with fluorescence and post-column cholesterol detection. BODIPY-LPS was monitored by excitation wavelength at 530 nm and emission wavelength at 550 nm and NBD-LPS at 465 and 535 nm, respectively. Cholesterol concentration in the column effluent was continuously monitored at 505 nm by enzymatic reaction with PAP reagent (Bio-Merieux, Marcy l’Etoile, France), in a reactor coil (1 m x 0.5 mm i.d.) at a flow rate of 0.1 ml/min. For the time-course experiments LPS was added to heparinized plasma (2 U/ml) for a final concentration of 24-40 µg/ml and incubated for 10 to 120 min at 37 °C prior to HPGC analysis. In some instances, peak fractions collected from sequential
chromatographic runs were pooled and concentrated for protein analysis using a Centricon-100 filter.

SDS-PAGE analysis of purified lipoprotein classes

Protein profiles of the individual lipoprotein classes isolated by HPGC, were analyzed by SDS-PAGE on pre-cast linear, 4-15 % (w/v) acrylamide gradient gels, containing a 4% (w/v) acrylamide stacking layer (Bio-Rad Hercules, USA) (41). Lipoprotein fractions were prepared for electrophoresis by heating for 5 min at 100 °C in sample buffer consisting of 50 mM Tris pH 6.8, containing 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromphenol-blue and 20 mM DTT. Separation was for 1.5 h at 15 mA in electrophoresis buffer consisting of 25 mM Tris, pH 8.3, 192 mM glycine and 0.1 % (w/v) SDS. Protein bands were developed by silver staining as described by Morrissey (42).

Results

Comparison of biological activity of fluorescent LPS and unlabeled LPS

The biological activities of all fluorescent LPS derivatives were compared with unlabeled LPS on the basis of TNF-α inducing capacity in whole blood stimulation assays. TNF-α production by labeled LPS (BODIPY-O111: B4, NBD-J5 and NBD-Re595) in the concentration range of 1 to 1000 ng/ml. were essentially similar to unlabeled LPS, within the limits of experimental error (Fig 1).

Competitive analysis of labeled and unlabeled LPS

Analysis of lipoprotein-associated fluorescence by HPGC revealed a linear response for all labeled LPS chemotypes (Fig. 2), indicating that no competitive inhibition by unlabeled LPS had occurred and that the lipoprotein binding characteristics of derivatized LPS is identical to unlabeled LPS.
Fig. 1. Comparison of the TNF-α inducing capacity of labeled or unlabeled O111:B4 (A), J5 (B) or Re595 (C) in whole blood from three healthy volunteers. LPS at concentrations of 1, 10, 100 and 1000 ng/ml whole blood, were incubated for 4 hours at 37 °C and TNF-α levels measured in duplicate. The values represent the average ± SD.
Fig. 2. Competition between labeled and unlabeled LPS for binding to plasma lipoproteins. (A) BODIPY-O111:B4 and (B) NBD-J5 and (C) NBD-Re595 show the distribution of labeled LPS lipoprotein in association with VLDL, LDL or HDL in plasma in the presence of unlabeled LPS for plasma concentrations approaching saturation (200, 300 and 200 μg/ml) for the three chemotypes, respectively. The LPS mixtures were added to the plasma samples and incubated for 20 minutes at 37 °C. Correction was done for the natural fluorescent background of the plasma components at the ex./emnm. Wavelengths used. Linear regression was used for the generation of the curves.
**Lipoprotein associated LPS**

**Characterization and purity of lipoproteins separated by size-exclusion chromatography**

Total plasma lipoproteins were completely separated into the main lipoprotein classes by HPGC on Superose 6 as demonstrated by a typical cholesterol profile in Fig. 3A. To verify the resolution of the separation, peak fractions were analyzed for apolipoprotein content. Apo A-I was found exclusively in the HDL fraction and apo B was only detectable in the LDL and VLDL peak fractions, indicating that no cross-contamination of the lipoprotein classes had occurred (data not shown). SDS-PAGE profiles of the isolated HDL, LDL and VLDL fractions, revealed multiple protein bands in addition to the normal apolipoprotein complement of each lipoprotein class. The molecular weights of a number of major bands associated with HDL, for example, correspond well with that of known lipid/LPS transport proteins such as LCAT, CETP, LBP and PLTP (Fig. 3B). Pooled HDL fractions were in fact shown to contain almost all of the plasma LCAT activity (data not shown). Separation of the lipoprotein classes by size exclusion chromatography under the conditions described in this work, yielded pure fractions containing particles with a unique and reproducible protein profiles comprising apolipoproteins, lipid/LPS transport proteins as well as a number of additional lipoprotein-associated proteins.

**Specificity of LPS binding**

To determine the binding specificity of LPS for lipoproteins, equal amounts of J5 LPS were incubated with delipidated or normal plasma and the LPS fluorescence profiles determined by HPGC (Fig. 4). The cholesterol profile of delipidated plasma demonstrates extremely low levels of residual cholesterol and a total absence of a typical lipoprotein profile, indicating that delipidation was complete and that the plasma is essentially devoid of lipid particles (Fig 4A). The LPS fluorescence chromatogram of lipoprotein-deficient plasma, displays an entirely different profile (Fig. 4B), and yielded approximately 5% of the total LPS fluorescence signal compared with that of normal plasma (Fig. 4B).
Fig. 3. (A) Chromatographic profile of the major plasma lipoprotein classes separated by HPGC with on-line cholesterol detection. The average molecular weights calculated from retention characteristics were 5 MDa for VLDL, 1MDa for LDL and 300 kDa for HDL. The horizontal bars indicate the lipoprotein fractions pooled for further analysis. SDS-PAGE analysis of these lipoprotein fractions with subsequent silver staining shows the protein composition of the lipoproteins (B). Lane M,: molecular weight in kDa. Lane A: VLDL, Lane B: LDL and Lane C: HDL. Volumes of 20 μl containing total of approximately 2 μg protein were loaded per well.
Fig. 4. LPS binding specificity of plasma lipoproteins. The chromatographic LPS cholesterol- (A) and fluorescence- (B) profiles of normal plasma (continuous lines) and delipidated plasma (dotted lines). Both the normal and delipidated plasma was incubated with NBD-15 LPS for 60 minutes at 37 °C and the fluorescence and cholesterol lipoprotein profiles determined as described in materials and methods.
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Distribution, saturation and LPS-binding capacity.

To determine the relative distribution of LPS among the lipoprotein classes under simulated physiological conditions, fluorescent LPS was incubated with aliquots of whole blood for a pre-selected (1 h) time interval and subsequently bound LPS was determined by HPGC. 93 – 99 % of the total LPS signal was found to co-elute with the lipoprotein peaks. The average distribution for all three LPS chemotypes in the HDL and LDL fractions was found to be essentially similar. HDL showed the highest, LDL intermediate and VLDL the lowest LPS binding in the LPS concentration range used (Table 1). Heat treatment and clarification of plasma (60 °C, 30 min; 12000 x g 20 min) prior to LPS addition resulted in a normal cholesterol profile, but severely reduced lipoprotein-associated fluorescence (data not shown) indicating that proteins participate in the loading of the particles with LPS. A chemotype-dependent variation in VLDL bound LPS was apparent. Re595 LPS appears to have a 2 and 4-fold higher preference for VLDL than J5 or O111:B4 LPS, respectively. A relatively small percentage of the total LPS signal was detected in the plasma protein fraction. LPS saturation analysis indicated a dose-dependent binding of O111:B4, J5 or Re595 LPS to the lipoprotein classes (Fig. 5). The total binding capacity of plasma lipoproteins for all LPS chemotypes examined, was found to be in excess of 200 μg/ml (Fig. 6A - C). HDL shows the highest binding-capacity for LPS and appears to be saturated at an Re595 LPS concentration of 200 μg/ml whole blood. Saturation was not achieved with O111:B4 or J5 LPS concentrations up to 200 μg/ml. Chemotype-dependent differences in saturation capacity are evident for VLDL and LDL.
Fig. 5. The chromatographic profiles showing a dose-response of LPS-lipoprotein association with three LPS chemotypes. A (O111:B4), B (J5) and C (Re-595) respectively. All main lipoprotein classes, protein indicated in figure A are also representative for figure B and C. The LPS concentrations used, are indicated in the different figures and were incubated for 1 hour at 37 °C. All chromatograms were corrected for the inherent fluorescence of plasma components. The data are representations of a number of independent experiments.
Fig. 6. The LPS binding capacity of plasma lipoproteins. Diagrams of lipoprotein associated LPS (A) BODIPY-O111:B4 and (B) NBD-I5 and (C) NBD-Re-595 showing a dose-dependent distribution of LPS-lipoprotein association on VLDL, LDL, and HDL in plasma obtained from a healthy volunteer. The values represent the mean ± 1 SD of duplicate results after correction of the natural fluorescent background of the plasma components at the used ex/em wavelengths. Non linear regression data fit was used for the generation of the curves.
**Lipoprotein associated LPS**

**Table 1.** Distribution of LPS among human lipoprotein classes.

<table>
<thead>
<tr>
<th>LPS</th>
<th>Conc. (µg/ml)</th>
<th>VLDL (%)</th>
<th>LDL (%)</th>
<th>HDL (%)</th>
<th>Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BODIPY-O111:B4</td>
<td>30</td>
<td>11 (±6)</td>
<td>25 (±7)</td>
<td>57 (±7)</td>
<td>7 (±5)</td>
</tr>
<tr>
<td>NBD-J5 (Re)</td>
<td>35</td>
<td>5 (±5)</td>
<td>29 (±10)</td>
<td>65 (±13)</td>
<td>1</td>
</tr>
<tr>
<td>NBD-Re595</td>
<td>30</td>
<td>19 (±4)</td>
<td>22 (±2)</td>
<td>58 (±5)</td>
<td>1</td>
</tr>
</tbody>
</table>

The relative distribution of LPS among lipoprotein classes in whole blood (WB) from healthy volunteers (n=10) after incubation for 1 h at 37 °C and analysis by high performance gel-permeation chromatography (HPGC). Values are expressed as means ± SD.

**Kinetics of LPS binding**

To determine the effect of incubation time on the LPS distribution among the lipoprotein classes, 4 different LPS concentrations (table 2) were added to plasma samples drawn at various time-points and the amount of lipoprotein-bound LPS was determined by HPGC (Fig. 7A – C). The data show that the loading of lipoprotein particles with O111:B4 LPS progressively increased to 40 min, with no further change in distribution on continued incubation (Fig. 7A). The loading of J5 or Re595 LPS however, was essentially complete at 10 min and a re-distribution of LPS from HDL to other lipoprotein classes was most evident at all LPS concentrations (table 2). A progressive decrease in HDL-bound J5 LPS coincided with an increase in LDL and VLDL signals (Fig. 7B). The 20% reduction in HDL-bound J5 LPS at 1 h appeared to be compensated for by a 70% increase in LDL-bound LPS (relative to the amount of bound LPS at 10 min). Similarly, an 18% reduction in HDL-associated Re595 LPS coincided with a 79% increase in VLDL-bound LPS relative to the amount of bound LPS at 10 minutes (Fig. 7C). Continued incubation with J5 LPS showed that the relative distribution of this chemotype changes with time, reaching equivalent amounts bound to LPS on HDL and LDL at 90 min and LDL-bound LPS exceeding HDL-bound LPS at 2 h. O111:B4 however, showed no decrease in HDL fluorescence signal within the 2 h incubation period (table 2).
Fig. 7. The LPS binding kinetics of plasma lipoproteins. Incubation of (A) 24 μg/ml BODIPY-O111:B4 and B) 35 μg/ml NBD-J5 and (C) 40 μg/ml NBD-Re-595. After incubation of individual plasma samples for t =10, 20, 40, 60 and 120 minutes at 37 °C, 60 μl 1:1 diluted plasma with TBS/Tween elution buffer was analyzed on HPGC as described before. All points represent peak areas corrected for inherent background fluorescence of plasma components at the used ex/emm. wavelength. Non linear regression data fit was used for the generation of the curves. The graphs are a representation of one of the 4 LPS concentrations used in these experiments which had all similar results.
Table 2: Overall changes of lipoprotein associated LPS distribution in time.

<table>
<thead>
<tr>
<th>LPS chemotype</th>
<th>Conc. Range (μg/ml)</th>
<th>Δ (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VLDL</td>
<td>LDL</td>
</tr>
<tr>
<td>O111:B4</td>
<td>6-12-24-36</td>
<td>38 (16)</td>
</tr>
<tr>
<td>J5</td>
<td>9-15-21-35</td>
<td>7 (12)</td>
</tr>
<tr>
<td>Re595</td>
<td>10-15-30-40</td>
<td>76 (8)</td>
</tr>
</tbody>
</table>

The percent change of LPS association in time (10–120 minutes) to the main lipoprotein classes relative to the amount of bound LPS at 10 minutes. Presented is the mean change ± (SD) per lipoprotein class of 4 different LPS concentrations for each LPS chemotype.

Discussion

In this study we present the overall distribution, saturation analysis and association kinetics of three biologically active labeled LPS chemotypes among the major lipoprotein classes using high performance gel-permeation chromatography (HPGC) for the quantitation of LPS binding. We examined the binding characteristics of three different LPS chemotypes that represent the range from smooth to deep rough phenotypes which may all be present in the outer membranes of wild-type bacterial pathogens depending on the growth stage and nutrient source. The E. coli serotype O111:B4 is a smooth LPS that contains long O-antigenic polysaccharide side-chains whereas the E. coli serotype J5 and S. typhimurium Re LPS that contain relatively short polysaccharide side-chains yield a rough and deep-rough colony morphology, respectively (35). Evaluation of the resolution of the lipoprotein separation, using nephelometric and SDS-PAGE analyses revealed the presence of apo A-I exclusively in the HDL fraction and apo B only in the LDL and VLDL fractions. With the use of monoclonal antibodies no apolipoprotein cross-reactivity in any of the lipoprotein classes was detected indicating that separation of all three lipoprotein classes by size-exclusion chromatography was complete. Lipoproteins isolated by differential ultracentrifugation generally show a much lower protein content of the HDL and LDL fractions and no detectable proteins within the VLDL fraction. Hence, these findings are in accordance with the observation that ultracentrifugation removes the majority of lipoprotein-
associated proteins (43), (28). Kunitake et al. have demonstrated that HDL loses substantial amounts of associated apo A-I and probably several other proteins which may be of importance in lipid/LPS transfer (28). SDS-PAGE analysis of the lipoprotein-associated proteins reveals multiple protein-bands in the <200 kDa region (Fig. 3B), confirming that, in addition to the major apolipoproteins, as well as LBP (13), PLTP (44), CETP (45) and LCAT, known to be associated with HDL, a number of specific proteins appear to be uniquely associated with all of the lipoprotein classes.

In this study, we demonstrate that an average of 60% of the fluorescent LPS signal was recovered in HDL, 25% in LDL and 12% in the VLDL fractions after incubation for 1 hour at 37 °C. Compared to previous results on the LPS distribution among the lipoprotein classes (16), (15), we demonstrate markedly lower levels of LPS associated with the plasma protein fraction (Table 1). Total delipidation of plasma resulted in a complete absence of lipoprotein classes as reflected by a 95% reduction in fluorescent LPS signal in the lipoprotein region of the chromatogram (Fig. 4), indicating that the binding of LPS by plasma lipoproteins is highly specific. Additionally, the dramatic decrease in LPS associated with the lipoprotein classes, present in heat-treated, clarified plasma (30 min at 60 °C: 12000 x g 20 min) provides additional evidence that ancillary proteins are involved in the “loading” of lipoproteins with LPS in an active process (unpublished observations).

A relatively higher amount O111:B4 LPS was recovered in the plasma protein fraction compared to J5- or Re595-LPS. This is probably a consequence of a faster “loading” mechanism of LPS for the more hydrophobic LPS chemotypes containing shorter O-antigen polysaccharide chains such as J5 and Re595 (Table 1). Furthermore we observed that LPS association is dose-dependent, even at LPS concentrations far exceeding those observed in clinical situations. Nonetheless, these high LPS concentrations have been employed in animal models of endotoxemia, and it is feasible that comparable or even higher LPS concentrations at a local focus of infection may be prevalent in the clinical setting.

Our results related to the kinetics of LPS association indicate a strict chemotype-dependence (Fig. 7). The polysaccharide chain length of LPS is presumably responsible for the velocity of the association: the shorter the polysaccharide chain, the more hydrophobic the LPS molecule in the order O111:B4 > J5 > Re-595, the higher the apparent affinity of LPS for the
Lipoprotein-associated LPS

Lipoprotein-phospholipid layer. During the 2 h incubation the overall amount of lipoprotein-bound LPS was nearly unchanged with time, indicating that the initial loading of LPS was complete within 10-20 minutes and was relatively chemotype independent. However, the amount of HDL-bound J5 LPS was seen to decrease with time and was accompanied by a corresponding increase in LDL-bound LPS. Similarly, a portion of the Re595 LPS fluorescence signal shifted from HDL to VLDL, indicating a re-distribution of these LPS chemotypes. Because it has been reported that the transport proteins LBP, CETP, and PLTP are able to bind and transfer LPS to lipoproteins (10), (13), it is tempting to speculate that these transfer proteins may also be responsible for the re-distribution of more hydrophobic LPS species with short polysaccharide-chains. It has been documented that clearing from the circulation of the rough type LPS such as J5 and Re595 is more rapid than the smooth type LPS such as O111:B4 (22). Therefore it may be feasible that the observed re-distribution towards LDL and VLDL of J5 or Re595 LPS, respectively, may play a role in the enhanced clearing of lipoproteins containing these LPS chemotypes from the circulation.

The question still remains as to why the inherently high endotoxin-binding capacity of lipoproteins, especially HDL, is not sufficient to diminish the effects of LPS during severe infection. This may be explained by two proposed pathways in the kinetics of LPS. Firstly, binding of LPS to peripheral blood mononuclear cells (46) may be more rapid than the second mechanism responsible for the loading of the lipoproteins with LPS. However, as we have demonstrated in this work, approximately 96% of added LPS is found to be associated with the lipoproteins within 10 to 20 minutes, a time period probably too short to induce an inflammatory response as proposed by Netea et al. (46). Further, the capacity of lipoproteins to promote the release of LPS from the macrophage surface has recently been described (47) which should favor a protective role for lipoproteins. Therefore it is feasible that, after the initial loading phase whereby LPS monomers are actively transferred from large miscellar LPS structures predominantly to HDL, the subsequent chemotype-dependent redistribution of LPS between the lipoprotein classes, may inadvertently result in the presentation of LPS to monocytes and macrophages whereby the inflammatory response is triggered.

In summary, binding of smooth and rough LPS to lipoprotein particles is specific and of relatively high affinity. The binding capacity of all LPS
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Chemotypes exceed concentrations observed in most clinical situations and added LPS is mainly associated with HDL particles. In addition, we describe a LPS-chemotype dependent re-distribution of HDL-associated LPS to LDL (J5-LPS) or VLDL (Re-LPS). This mechanism may have important implications in the magnitude and duration of the inflammatory reaction initiated by the presence of LPS in the host.

Acknowledgments

We would like to thank K. Bakhtiari and H.P. van Barreveld for excellent technical assistance and we are indebted to Dr. B. J. Appelmelk (Department of Medical Microbiology, Vrije Universiteit, Amsterdam) for critical reading of the manuscript.

References

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