Lipoproteins in innate immunity
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Endotoxin sequestering characteristics of HDL subtypes

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Invading Gram-negative or Gram-positive pathogens release toxic cell wall components such as lipopolysaccharides (LPS) or lipoteichoic acids (LTA), respectively, which have potent inflammatory properties. Lipopolysaccharide binding protein (LBP), a member of a family of lipid transport proteins known to be associated with high density lipoproteins (HDL), has been implicated in the loading of HDL with endotoxin and in the attenuation of the host response to infection. We have recently shown that total human plasma HDL has a high binding capacity for LPS or LTA and in this study, we determined the HDL subfraction primarily responsible for endotoxin sequestration \textit{ex vivo}. Fresh plasma was incubated with three different LPS chemotypes or LTA fluorescently labeled with BODIPY or NBD, the major lipoprotein classes pre-fractionated with 7.5\% (w/v) PEG 6000 and analyzed for endotoxin binding using high performance gel-permeation chromatography. Further characterization of the isolated lipoprotein fractions was by immunochemical analysis, lipoprotein electrophoresis and 1-D and 2-D polyacrylamide gel-electrophoresis.

LPS or LTA were recovered in a dose-dependent fashion in the PEG-soluble HDL fraction with alpha-mobility. A small amount of J5 or Re595 LPS (<10\%) was found to be associated with a PEG-precipitable, lipid-poor fraction with pre-beta mobility. The overall protein composition of this fraction showed similarities with \(\alpha\)-HDL and accounted for 20 ± 15\% of the total HDL protein content.

We conclude that cholesterol-rich HDL with \(\alpha\)-mobility accounts for the majority of the endotoxin-binding potential of HDL. We suggest that the PEG precipitable fraction represents a HDL sub-population with poor affinity for endotoxin.
Introduction

Infection of the host by Gram-negative or Gram-positive pathogens and subsequent release of membrane components such as lipopolysaccharides (LPS) or lipoteichoic acids (LTA), respectively, are the initiating events of severe sepsis. Depending on the extent and duration of the infection and the efficacy of host immune responses, acute systemic inflammation may lead to cytokine-mediated septic shock and high mortality (1), (2). It has recently been demonstrated that plasma lipoproteins play an important role in innate immunity against bacterial infection (3), (4).

Profound changes in the plasma concentration and lipid composition of all lipoproteins (5), (6) and in the apolipoprotein content of high density lipoprotein (HDL) (7), (8) have been observed in sepsis, and these are expected to importantly modulate the host response. Previous studies have shown that LPS and LTA are sequestered by lipoproteins from various animal species, (9), (10), (11). In this regard, we have recently demonstrated that of the major human lipoprotein classes, HDL has the highest affinity and binding capacity for LPS (12) or LTA (this thesis). The changes in lipid composition of HDL and LDL in sepsis, as well as the transfer of LPS to and from lipoproteins is mediated by a family of lipid transport proteins, that includes phospholipid transfer protein (PLTP), cholesteryl ester transfer protein (CETP) and bactericidal/permeability increasing protein (BPI) (10).

Lipopolysaccharide binding protein (LBP), has been implicated in the loading of HDL (13) with endotoxin and alters the host response to infection in animal models (14), (15), (16), (17) and in humans (18). In addition, phospholipid transfer protein (PLTP) has been described as having the ability to transfer LPS among the lipoprotein classes (19), (20).

In this study, we wished to determine the HDL subtype primarily responsible for endotoxin binding. We used mild lipoprotein separation methods to preserve to particle integrity (21) such as high performance gel-permeation chromatography (HPGC), lipoprotein precipitation with 7.5% (w/v) PEG-6000 and immuno-affinity chromatography. The LPS-fluorescence recovery in total HDL, Lp A-I and Lp A-I/II HDL subtypes was measured. Further, the LPS and LTA distribution in the PEG-soluble and the PEG-precipitable plasma fractions were determined. Finally, we compared the protein composition of PEG-precipitable-, PEG-soluble HDL and immuno-affinity purified- and by two-
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dimensional electrophoresis in combination with MALDI-TOF fingerprint mass spectrometry.

Materials and Methods

Materials

Lipopolysaccharides of the highest purity were obtained from commercial sources. *Escherichia coli* O111:B4 LPS was from Sigma Chemical Co. (St Louis, MO), *E. coli* J5 (Re) and *Salmonella typhimurium* Re595 LPS were from List Biological Laboratories (Campbell, CA, U.S.A.). *Staphylococcus aureus* LTA was from Sigma Chemical Co. (St Louis, MO, U.S.A.). The fluorescent labels NBD-F and BODIPY-R6G were from Molecular Probes Inc. (Eugene, OR, U.S.A.). Certified pyrogen-free distilled water from Ecotainer (Braun Medical AG, Melsungen, Germany) was used throughout the experiments. Polyethylene glycol-6000 (PEG-6000) was from Aldrich Sigma (Steinheim, Germany). The polyclonal antibodies anti-Lp A-I and anti-Lp A-I/II were obtained from Dr. J.C. Fruchard (Dept. of Biochemistry, University of Lille, France). Apo B and apo A-I levels of the lipoprotein fractions were measured by an automated turbidimetric assay using the APA and APB kit on an Array Protein System Nephelometer (Beckman, Mijdrecht, Netherlands).

Methods

**Incubation of plasma with endotoxin**

Plasma samples, obtained from six healthy volunteers after informed consent, were incubated with three different fluorescent LPS chemotypes: *E. coli* O111:B4 (20 mg/L), J5 or *S. minnesota* Re595 (30 mg/L) or *S. aureus* LTA (40 mg/L). Fluorescently labeling of LPS or LTA with BODIPY or NBD was done as previously described (12). Samples were incubated for 1 hour at 37 °C and processed immediately.

**Selective lipoprotein precipitation**

Fresh plasma was fractionated by addition of PEG 6000 to 7.5% (w/v) essentially according to Vikari (22), and analyzed for endotoxin binding. Briefly, 200 µl of 45% (w/v) PEG stock solution, pH 7.4 was added to 1.0 ml citrated plasma and incubated for 15 minutes at room temperature. The samples were swirled every 2 minutes and subsequently centrifuged at 2000 rpm for 8 minutes in an Eppendorf microfuge at room temperature. The supernatant was aspirated and stored until further process. The precipitate was resuspended in 1 ml TBST buffer (10 mM Tris, 150 mM NaCl, pH 7.4 containing 0.005% v/v Tween-20) and stored at -80 °C until further analysis.

**High performance gel-permeation chromatography (HPGC)**

Lipoprotein separation by size-exclusion chromatography was as previously described (12). Briefly, lipoprotein fractions were isolated by HPGC. Injection was carried out with 100 µl
LPS sequestering by HDL subtypes

Aliquots of sample (citrated plasma, PEG-supernatant or re-solubilized PEG-precipitate) previously diluted with an equal volume of TBST on a Superose 6 HR 10/30 column (Pharmacia Biotech Inc., Uppsala, Sweden) and developed as before with in-line fluorescence and cholesterol detection. Peak fractions containing well-separated lipoproteins were collected and concentrated with Centricon 100 filters to approximately 100 μl. Samples were processed immediately or were frozen in liquid nitrogen and stored at −80 °C until further analysis.

Lipoprotein gel electrophoresis

Native lipoprotein gel-electrophoresis was done using a lipoprotein electrophoresis kit (LIPO-GEL, Beckman Coulter) using a Paragon electrophoresis unit (Beckman Coulter, Beckman, Fullerton, USA). Briefly, 10 μl undiluted lipoprotein samples of fractions or plasma were applied to loading slots in a pre-poured agarose gel on a carrier film. After electrophoretic separation at 200 V for 90 min at room temperature in barbital-buffer (10 mM 5.5-diethylbarbituric acid, 50 mM 5.5-diethylbarbituric acid sodium salt, pH 8.6), the gels were fixed (10% acetic acid/60% methanol/30% water mixture) for 30 minutes and dried. Lipids and proteins were visualized by staining with Sudan black, respectively.

Electrograph Microscopy

For cryo-electron microscopy, 400-mesh grids were dipped into the samples of isolated lipoproteins, blotted with filter paper, and vitrified in liquid ethane with a gravity-driven guillotine kept at −196 °C in liquid nitrogen. Samples were examined and photographed with an EM420 electron microscope (Philips, Eindhoven, The Netherlands) at 100 kV at various magnifications.

1-D polyacrylamide gel-electrophoresis

The protein profiles of the individual lipoprotein classes isolated by HPGC were analyzed by polyacrylamide gel-electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) on pre-cast 4-15% (w/v) polyacrylamide gradient gels (Bio-Rad Hercules, USA) (23). 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, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol-blue and 20 mM DTT. Protein bands were visualized by Coomassie Brilliant Blue staining.

2-D polyacrylamide gel-electrophoresis

Each plasma sample (equivalent to 300 μg of protein) was treated with 1-D buffer (40 mM Tris, pH 7.4, containing 9.0 M urea, 2% w/v CHAPS, 2 mM DTT in a final volume of 250 μl. Samples were incubated for 1 h at room temperature before application onto Immobiline dry strips (Amersham Pharmacia Biotech, Uppsala, Sweden). Passive rehydration was allowed to continue overnight.

The first dimension was for 40000 V h in a Multiphor II electrophoresis unit (Amersham Pharmacia Biotech. Inc., Uppsala, Sweden). Subsequently the strips equilibrated with 1% (w/v) DTT for 15 minutes at RT followed by a second equilibration with 2.5% (w/v) iodoacetamide without DTT in SDS equilibration buffer (50 mM Tris, pH 6.8, 6 M Urea, 30% (v/v) glycerol and
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2.5% (w/v) SDS). Finally, the strips were loaded onto 12% (w/v) polyacrylamide separation gels or 4-15% (w/v) polyacrylamide gradient gels containing 0.1% (w/v) SDS. Electrophoretic loading was for 60 min at 20 mA/gel and separation for 3-4 hours at 60 mA/gel in a Hoefer Electrophoresis System (Pharmacia, Uppsala, Sweden). Gels were fixed with a solution containing 7.5% (v/v) acetic acid and 20% (v/v) isopropanol and proteins were visualized by silver staining (24). For qualitative analysis, the gels were vacuum dried between cellophane and protein bands excised for mass spectrometry.

Mass spectrometry

For MALDI-TOF analysis, protein-containing gel slices were S-alkylated with iodoacetamide, digested with trypsin (sequencing grade, Roche Molecular Biochemicals.), and extracted according to Shevchenko et al. (25). Only peptides eluted with 20 mM ammonium carbonate were used in the analysis. After drying in a vacuum centrifuge, the polypeptides were dissolved in 10 µl of a solution containing 1% (v/v) formic acid and 60% (v/v) acetonitrile. Eluted peptides were mixed 1:1 (v/v) with a solution containing 52 mM α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemie B.V.) in 39% (v/v) ethanol, 49% (v/v) acetonitrile, 2% (v/v) tri-fluoric acid and 1 mM ammonium acetate. Prior to preparation of the solution, the α-cyano-4-hydroxycinnamic acid was washed briefly with acetone. The mixture was spotted onto the target and allowed to dry at room temperature. Reflectron MALDI-TOF spectra were acquired on a Micromass TOF Spec HT (Wythenshawe, UK). The resulting peptide spectra were used to search several non-redundant protein databases.

Isolation of HDL subtypes immuno-affinity chromatography

Rabbit anti-human polyclonal antibodies against two subtypes of HDL (Lp A-I and Lp A-I/II) (26) were coupled to Sepharose 4B beads according to the manufacturer’s instructions (Pharmacia Biotech Inc., Uppsala, Sweden) and HDL particles isolated as previously described (27). Briefly, HDL particles were isolated from citrated plasma by loading 0.5 ml plasma onto the affinity column washing extensively with TBS (3 to 4 column volumes) and eluting with 3 M sodium isothiocyanate followed by immediate dialysis. The samples were concentrated with Centricron-30 filters (Beverley, MA, USA) to 0.5 ml and stored at –80 °C until further analysis.

Results

Analysis of lipoproteins after PEG-6000 precipitation of VLDL/LDL

The major lipoprotein classes of six healthy volunteers (lanes 2-7) were fractionated with 7.5% (w/v) PEG-6000 and subjected to LIPO GEL lipoprotein electrophoresis with subsequent lipoprotein staining (Fig. 1). Compared to total plasma, the beta (precipitate) and alpha (supernatant) migrating particles were completely separated, indicating that the entire HDL population was separated from VLDL and LDL.
LPS sequestering by HDL subtypes

![Lipoprotein profile](image)

**Fig. 1.** Lipoprotein profiles of total and PEG-fractionated plasma from six volunteers (lane 2-6). PEG-precipitate and PEG-supernatant refers to 7.5 % (w/v) PEG-precipitable and PEG-soluble material. The arrow indicates the origin of sample application. Lipid staining was with Sudan Black.

![Chromatographic profile](image)

**Fig. 2.** Chromatographic profiles of LPS and cholesterol recovery in lipoprotein fractions isolated in various ways: (A) An example of HPGC analysis with fluorescence and cholesterol detection of total plasma, PEG-soluble, LP A-I and Lp A I/II HDL, and PEG-precipitable lipoprotein fractions (Panel A). The scales of the fluorescence and cholesterol signals are relative to 100% with total plasma. UV profile (280 nm) of the PEG-precipitable plasma fraction (Panel B). The bar indicates the PEG-precipitable protein fraction with the same chromatography retention time as HDL.

Immunonephelometric analysis confirmed that the supernatant was free of apo B containing particles. A minor quantity of apo A-I was detected in the
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precipitate (Table 1) indicating that a sub-population of HDL appeared to be precipitated by PEG at the concentration used for the complete separation of cholesterol-rich HDL from LDL and VLDL.

Table 1. The apolipoprotein composition of the PEG-precipitated plasma fraction compared to total plasma ± SEM from 6 volunteers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Apo A-I (SD) mg/L</th>
<th>Apo B (SD) mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Plasma</td>
<td>1327 (150)</td>
<td>817 (189)</td>
</tr>
<tr>
<td>S</td>
<td>1280 (165)</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>76 (6)</td>
<td>715 (189)</td>
</tr>
</tbody>
</table>

Supernatant (S) and precipitate (P) refers to 7.5% (w/v) PEG-6000 soluble and precipitable components of plasma, respectively.

High performance gel chromatography analysis (HPGC) of the lipoprotein fractions

Fig. 2A is an example of the HPGC analysis with fluorescence and cholesterol detection of total plasma PEG-soluble and PEG-precipitable lipoproteins, Lp A-I and Lp A-I/A-II. Endotoxin was recovered in a dose-dependent fashion in the PEG-soluble HDL (S/N). Lp A-I HDL and the Lp A-I/A-II HDL subtypes. Comparison of the LPS fluorescence signal with the cholesterol distribution revealed that LPS was mainly associated with the cholesterol containing fractions.

<table>
<thead>
<tr>
<th>O111:B4</th>
<th>J5</th>
<th>Re</th>
<th>LTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects P S</td>
<td>P S</td>
<td>P S</td>
<td>P S</td>
</tr>
<tr>
<td>6</td>
<td>5.1 (2.2)</td>
<td>94.9 (2.2)</td>
<td>6.1 (1.7)</td>
</tr>
</tbody>
</table>

Table 2. The relative distribution of endotoxin (SD) ± SEM between the PEG precipitable (P) and PEG-soluble (S) HDL-sized fractions after incubation with different fluorescently labeled LPS chemotypes: E. coli O111:B4 or J5 or S. minnesota Re 595 LPS or S. aureus LTA. The relative fluorescent peak areas, that represent endotoxin binding to HDL, were determined after HPGC analysis.
LPS sequestering by HDL subtypes

The recovery of LPS in the PEG-precipitable fraction (P), which contains VLDL and LDL, was dose-dependent. However, a minor endotoxin fluorescence signal (< 10%) was observed in the protein fraction associated with a PEG-precipitable, lipid-poor particle which appeared to have pre-beta-mobility (not shown) and HDL migration behavior during HPGC (Fig. 2B) as revealed by detection at 280 nm. The overall LPS and LTA distributions between the PEG-soluble and the PEG-precipitable HDL fractions in plasma from six volunteers are illustrated in Table 2. No differences in LPS or LTA distribution in the PEG-soluble or PEG-precipitable fraction were observed in the plasma samples from male or female subjects. The quantitative recovery of LPS or LTA in the PEG-soluble fraction was 94% and 89%, respectively. However, 5 to 11% was detectable in the PEG-precipitable fraction. This protein-rich fraction was isolated and subjected to electron microscopy (Fig. 3).

Fig 3. Electron microscopy of PEG-precipitable HDL-like particles (Panel A) and PEG-soluble HDL (Panel B). The bar corresponds to 10 nm.

This revealed that these particles were indeed of similar size compared to HDL (Fig. 3A), but the core of the PEG-precipitable particles appeared to be less dense compared to the PEG-soluble HDL particles (Fig. 3B). The total protein of these particles accounted for 20 ± 15% of the total HDL protein.

Protein composition of isolated HDL fractions
In order to further characterize the protein content of the PEG-precipitable HDL fraction, the protein profile was compared with that of the PEG-soluble HDL using SDS-PAGE. Additional, analysis by 2-D
electrophoresis on these two sub-populations of HDL and the LP A-I HDL-subtype was performed.

The protein stain of the PEG-precipitable particle, separated by 1-D electrophoresis, showed a remarkably similarity to the protein profile of HPGC isolated cholesterol-rich HDL (Fig 4). In addition, 2-D electrophoresis revealed only minor differences in the protein profiles of the PEG-soluble HDL (Fig. 5A), the PEG-precipitable particle (Fig. 5B) and LP A-I HDL (Fig. 5C). A significant degree of IgG contamination typical for a 2-D gel of plasma was apparent. The immuno-affinity isolation, although highly specific for HDL still revealed the presence of a small amount of IgG. This indicates that a small amount of IgG either co-elutes or is associated with HDL. Analysis of the protein composition of PEG-precipitable HDL by mass spectrometry showed that the main identified constituents of the particle were haptoglobin, IgG, serotransferrin and apo A-I, whereas in the PEG-soluble- and LP A-I HDL particles other HDL associated proteins were evident (Table 3).
Fig. 5. 2-D electrophoresis patterns of PEG-soluble HDL (Panel A) with the corresponding 1-D pattern to the right, PEG-precipitable protein fraction (Panel B) and Lp A-I HDL after immunaffinity isolation (Panel C). For the protein identifications see table 3.
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Table 3. Summary of proteins identified by MALDI-TOF analysis in the various HDL preparations as depicted in Fig. 5.

<table>
<thead>
<tr>
<th>Spot no</th>
<th>Protein</th>
<th>Spot no</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Apo C-II</td>
<td>11</td>
<td>Apo J</td>
</tr>
<tr>
<td>2</td>
<td>Apo C-III</td>
<td>12</td>
<td>Apo A-IV</td>
</tr>
<tr>
<td>3</td>
<td>Apo A-II</td>
<td>13</td>
<td>Albumin</td>
</tr>
<tr>
<td>4</td>
<td>Haptoglobin α-chain</td>
<td>14</td>
<td>Serotransferrin</td>
</tr>
<tr>
<td>5</td>
<td>Apo A-I</td>
<td>15</td>
<td>IgG Heavy, Intermediate and Light chain</td>
</tr>
<tr>
<td>6</td>
<td>Pro apo A-I</td>
<td>16</td>
<td>α1-β-glycoprotein</td>
</tr>
<tr>
<td>7</td>
<td>Apo D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Apo J</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Haptoglobin β-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Apo E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Using PEG precipitation, high performance gel-permeation chromatography and immuno-affinity chromatography, the recovery of LPS or LTA in specific HDL subtypes was investigated. The majority of the added fluorescent endotoxin was recovered in the cholesterol-rich HDL subtype, which is 7.5% PEG-soluble, or the immuno-affinity purified HDL particles, all representing HDL with α-mobility. Hence, endotoxin is sequestered by HDL particles that contain a full complement of lipids and phospholipids, which suggests that a phospholipid cholesterol-ester matrix is the most optimal environment for endotoxin binding. Remarkably, the three LPS chemotypes and the LTA preparation were all recovered in lipid-rich HDL, indicating that a common chemical moiety, namely the fatty acid side-chains in the lipid-A part of LPS or the two fatty acid chains of the LTA molecule determines the physical properties necessary for incorporation into the phospholipid monolayer of HDL. However, a minor part of LPS was recovered in a lipid-poor PEG-precipitable particle with the same chromatographic retention as mature HDL from total plasma or in the PEG-soluble HDL fraction. EM analysis demonstrated that these particles were indeed of approximately the same size as HDL. We initially assumed that these lipid poor HDL-like particles could be of pre-β HDL origin, but preparations of these particles failed to induce cholesterol efflux in vitro in a cholesterol efflux assay (28) (data not shown).
Further, in two of the six subjects, LBP was immunologically detected in this fraction (data not shown). Recently, several unique HDL subtypes have recently been described, including apo J or apo D containing HDL, very high density HDL (VHDL) (29) and FALP (particles containing apo A-I, phospholipids and Factor H-related proteins) (30), (31). It is possible that FALP particles were present in the PEG-precipitable protein fraction. Although the size and molecular weight (250 kDa) of the PEG-precipitable HDL-like particles was comparable with FALP, the protein composition was more complex according to 2-D analysis. The fact that haptoglobin, an acute phase protein which is also involved in lipid metabolism (32), was found to be associated with PEG-precipitable HDL combined with the presence of HDL-specific apo A-I, leads us to the suggestion that this new particle is indeed of HDL origin and may in fact be a steady-state HDL remnant population in the process of being cleared from the circulation.

In summary, we can conclude that plasma HDL with α-mobility accounts for the majority of the endotoxin sequestration activity of plasma lipoproteins. Additionally, we demonstrate a new HDL sub-population that apparently plays a minor role in LPS sequestration, but may be an integral part of the HDL catabolic pathway.

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