Lysophosphatidylcholine Is Involved in the Antigenicity of Oxidized LDL

Ruihua Wu, Yui Hui Huang, Liselotte Schäfer Elinder, Johan Frostegård

Abstract—Lysophosphatidylcholine (LPC) is formed by hydrolysis of PC in low density lipoprotein (LDL) and cell membranes by phospholipase A₂ or by oxidation. Oxidized (ox) LDL activates endothelial cells, an effect mimicked by LPC. oxLDL also has the capacity to activate T and B cells, and antibody titers to oxLDL are related to the degree of atherosclerosis. The antigen in oxLDL responsible for its immune-stimulatory capacity is not well characterized, and we hypothesized that LPC was involved. We demonstrate herein the presence of antibodies against LPC, both of the IgG and IgM isotype, in 210 healthy individuals. This antibody reactivity was not specifically related to oxidation of the fatty acid moiety in LPC, since LPC containing only palmitic acid showed antibody titers equivalent to those of LPC containing unsaturated fatty acids. Antibody titers to PC were low compared with LPC, and hydrolysis of PC at the sn-2 position is thus essential for immune reactivity. There was a close correlation between anti-oxLDL and anti-LPC antibodies. Furthermore, LPC competitively inhibited anti-oxLDL reactivity, which indicates that LPC may explain a significant part of the immune-stimulatory properties of oxLDL. LPC, being a lipid, is not likely to be an antigen itself. Instead, LPC could form immunogenic complexes with peptides, which may induce and potentiate immune reactions in the vessel wall. This study adds to the evidence that LPC is an important component of oxLDL and emphasizes the potential role of phospholipase A₂ in atherosclerosis. (Arterioscler Thromb Vasc Biol. 1998;18:626-630.)

Key Words: lysophosphatidylcholine • antibodies • oxidized LDL • autoimmune diseases • atherosclerosis

Anti-phospholipid antibodies have been found in a variety of inflammatory and autoimmune diseases such as SLE and are related to cardiovascular disease. Antibodies have been shown to be directed mainly against CL, a diphosphatidylglycerol, as well as phosphatidylethanolamine and phosphatidylserine. It is generally believed that phospholipids with antigenic properties bind to plasma proteins like β2-glycoprotein, thereby creating neoantigens, which are recognized by the so-called anti-phospholipid antibodies. Patients with significant amounts of anti-phospholipid antibodies may develop autoimmune thrombocytopenia and have an increased risk for thrombotic events.

According to a leading hypothesis, oxLDL plays a pivotal role in atherosclerosis. The early stages of atherosclerosis are characterized by the presence of activated macrophages and T lymphocytes, and oxLDL may play a role in this activation. Antibodies against oxLDL are present both in healthy individuals and in atherosclerotic plaques and are related to atherosclerosis progression. Furthermore, anti-oxLDL antibodies have been correlated with titers against CL in SLE patients. One of the active components of oxLDL is LPC, which may be formed during oxidation of LDL or from PC by enzymes with PLA₂ activity. Recently we identified secretory PLA₂ type II (ie, sPLA2-II, nonpancreatic type) expression and activity in both normal and atherosclerotic arterial walls, being mainly expressed by medial smooth muscle cells. This finding opens the possibility that significant amounts of LPC may be generated in the arterial wall by hydrolysis of phospholipids in retained LDL. In several autoimmune diseases like rheumatoid arthritis and SLE, elevated plasma PLA₂ activity has been demonstrated. Therefore, in these disorders, elevated amounts of LPC could be produced in the blood compartment. On the basis of this evidence, we wondered whether antibodies were produced in vivo against LPC. In the present article we present evidence that antibodies against LPC are present in normal, healthy individuals and that their titers can be correlated closely with antibody titers to oxLDL.

Methods

Study Group

Sera from the blood bank at the Karolinska Hospital was used in this study and consisted of sera from 210 healthy volunteering blood donors with a mean age ± SD of 46.6 ± 11.5 years. There were 150 males (46.81 ± 9.32 years) and 60 females (46.21 ± 13.52 years).

Lipids and Reagents

1-α-LPC (from egg yolk type 1, produced by PLA₂ treatment), 1-α-16:0 (LPC 16:0, also produced by PLA₂ treatment), 1-α-PC (from egg yolk type 1), and CL (from bovine brain) were from Sigma Chemical Co. LDL was isolated from the plasma of healthy donors by sequential preparative ultracentrifugation in a Beckman 50.3 Ti fixed-angle rotor (Beckman L8–80 ultracentrifuge) for 48 hours at 1°C and collected in the density interval 1.025 to 1.050 g/mL. The protein content was determined according to the method of Lowry et al.
adjusted to 200 μg/mL. The LDL was dialyzed against PBS (pH 7.4) for 24 hours and then oxidized by exposure to 5 μmol/L CuSO₄ for 18 hours at 37°C. This procedure has previously been shown to result in extensive oxidation of LDL. Antigen PPD (2.5 mg/mL) was used to coat the ELISA plates (Costar 2581). The plates were kept at 4°C overnight, washed three times with PBS containing 0.05% Tween-20, and then blocked with 20% ABS-PBS for 2 hours at room temperature. The plates were then incubated with 100 μL serum, diluted 1:30 in 20% ABS-PBS at 4°C overnight. Antibodies against CL, PC, LPC, and LPC 16:0 were analyzed essentially as described, with CL as the antigen. In brief, Titertek 96-well polyvinylchloride microplates (Flow Laboratories) were coated with 50 μL per well of 50 μg/mL lipid dissolved in ethanol and allowed to dry overnight at 4°C. Blocking was accomplished with 20% ABS-PBS for 2 hours. Serum samples (50 μL each), diluted 1:30 in 20% ABS-PBS, were added to each well. Antibody reactivity to the EB-Na component in Epstein-Barr virus was detected with an ELISA according to the manufacturer’s instructions (Blotest). In brief, 100 μL per well of 50 ng/mL EB-Na was used to coat the ELISA plates (Costar 2581). The plates were then incubated with 100 μL serum, diluted 1:30 in 20% ABS-PBS at 4°C overnight. After three washes with PBS, the plates were incubated with 50 μL/m of alkaline phosphatase–conjugated goat anti-human IgA (Sigma A-3400), diluted 1:10000; IgG (Sigma A-3150), diluted 1:9000; or IgM (Sigma A-3275), diluted 1:7000 with PBS at 37°C (for 2 hours. After three washes, 100 μL of substrate (phosphatase substrate tablets, Sigma 104; 5 mg in 5 mL diethanolamine buffer, pH 9.8) was added. The plates were incubated at room temperature for 30 minutes and read in an ELISA Multiskan Plus spectrophotometer at 405 nm. Each determination was done in triplicate. The coefficient of variation between triplicate tests was <5%. Cross-reactivity Between Antibodies to LPC and oxLDL To investigate whether there was any immunological cross-reactivity between the antibodies tested, competition assays were performed. Sera at a dilution that yielded 50% of maximal binding to the compound coated were preincubated with different concentrations of oxLDL, LPC, CL, or EB-Na. The sera were incubated overnight with different competitors at 4°C, and inhibition of binding to oxLDL was tested. The percentage of inhibition was calculated as follows: percent inhibition = \( \frac{OD_{\text{control}} - OD_{\text{with competitor}}}{OD_{\text{control}}} \times 100 \), where OD is the optical density in nanometers.

**Statistical Methods**

Conventional methods were used for calculation of means and SDs. Parallelism between dilution curves was determined by correlation coefficient analysis and Fisher’s test. Simple regression was used to analyze the correlations between the antibodies and between age and the antibodies.

### Results

**Antibody Titers Against LDL, oxLDL, CL, and LPC**

The levels of IgA, IgG, and IgM antibodies against LDL, oxLDL, CL, and LPC were determined. In general, antibodies of the IgA isotype to these antigens were very low (data not shown). The levels of both IgG and IgM antibodies are shown in Table 1. Antibodies against oxLDL were significantly higher (P<.001) than those against LDL. The IgG and IgM antibody titers against both LPC and CL were clearly detectable in the group as a whole, although marked individual variation was noted. There was no correlation between antibody titer and age (data not shown).

**Analysis of Antibodies Against LPC**

To identify the characteristic feature of LPC that induces antibody formation, we tested the antigenicity of PC obtained from the same source as LPC (egg yolk) and that of LPC 16:0, i.e., LPC containing only saturated palmitic acid in the sn-1 position. For this purpose, 20 serum samples with the highest antibody titers against LPC were chosen. The levels of antibodies against PC, LPC, and LPC 16:0, are shown in Fig 1. The levels of antibodies against LPC (IgM, 0.320±0.092; IgG, 0.335±0.2; mean±SD) and against LPC 16:0 (IgM, 0.342±0.14; IgG, 0.325±0.15) were both significantly higher (P<.001) than the levels of antibodies against PC, which were quite low (IgM, 0.11±0.077; IgG, 0.075±0.11). There was no significant difference between the levels of antibodies against

### Table 1. Antibody Titers Against LPC, oxLDL, LDL, and CL in 210 Healthy Individuals

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Range</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-LPC</td>
<td>IgG</td>
<td>0−1.027</td>
<td>0.25±0.119</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0.017−0.707</td>
<td>0.203±0.097</td>
</tr>
<tr>
<td>Anti-oxLDL</td>
<td>IgG</td>
<td>0.017−0.777</td>
<td>0.149±0.11</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0−1.663</td>
<td>0.324±0.23</td>
</tr>
<tr>
<td>Anti-LDL</td>
<td>IgG</td>
<td>0−1.235</td>
<td>0.093±0.105</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0−0.764</td>
<td>0.177±0.091</td>
</tr>
<tr>
<td>Anti-CL</td>
<td>IgG</td>
<td>0−0.873</td>
<td>0.242±0.132</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>0−0.765</td>
<td>0.134±0.074</td>
</tr>
</tbody>
</table>

\( OD_{405} \) indicates optical density at 405 nm.

**Figure 1.** Box-and-whisker plots of antibody titers (optical density at 405 nm) to LPC from egg yolk, LPC 16:0, and PC (from egg yolk). Both IgG and IgM isotypes are shown. The 20 sera with the highest antibody titers against LPC were used.
LPC and those against LPC 16:0. There was a strong, linear correlation between antibody titers against LPC and LPC 16:0 (IgG, $r = .841$, $P < .0001$; IgM, $r = .784$, $P < .0001$). The distribution of antibody titers against LPC for the whole group was almost normal, as shown in Fig 2.

To further characterize antibody reactivity to LPC, different dilutions of sera from four high- and four low-titer individuals were investigated. The curves for the individuals with high titers were parallel, since correlation coefficients between them were $>.91$ and $P$ values were $<.002$ in all comparisons between the curves (Fig 3).

The correlation between antibodies against LPC and antibodies against LDL, oxLDL, and CL are shown in Table 2. There was a significant correlation ($P < .0001$) between antibody titers against oxLDL and LPC for both the IgG and IgM isotypes. Likewise, antibody titers were correlated between LPC and CL, particularly those of the IgM isotype ($P < .0001$). Other significant correlations were found between LDL and oxLDL (data not shown). No significant association was found between IgG antibody titers against oxLDL and CL, but for IgM, there was a comparatively weak correlation (Table 2).

Cross-reactivity of Antibodies to LPC and oxLDL
To study possible cross-reactivity between the antibodies, we performed competition experiments with LPC, CL, and oxLDL at a dilution that yielded 50% of maximal binding to oxLDL. As a control, the unrelated antigen PPD was used. Fig 4 shows that LPC and oxLDL had the same capacity to inhibit serum binding to oxLDL-coated plates ($P < .01$ when compared with PPD; $P < .05$ when compared with CL). CL had an intermediate capacity in this respect ($P < .05$ when compared with PPD), whereas PPD showed no inhibitory effect.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Distribution of antibodies to LPC (egg yolk) in healthy individuals ($n = 210$). Antibody titers to LPC were determined by ELISA and the concentration expressed as optical density values at 405 nm.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Antibody reactivity to LPC. Sera from 8 individuals, 4 with high and 4 with low antibody titers, were diluted as indicated. Antibody titers of IgG type to LPC were determined by ELISA and the concentration expressed as optical density values at 405 nm. Results are mean of triplicate determinations.

To exclude the possibility that LPC has a general ability to interfere with antibody binding, the antibody reactivity (very common in the population) to EB-Na, a component of Epstein-Barr virus, was studied. When ELISA plates were coated with EB-Na, oxLDL, or LPC and EB-Na was used as the competitor, antibody binding to EB-Na itself was strongly reduced, whereas there was no effect on binding to LPC or oxLDL (Fig 5A). However, when LPC instead of EB-Na was used as a competitor, binding to LPC and oxLDL was strongly reduced, whereas binding to EB-Na was not influenced (Fig 5B).

**Discussion**
A new finding of the present study is that healthy subjects produce antibodies against LPC, a major component of oxLDL. Lysophosphatidylcholine and OxLDL Antigenicity by guest on October 15, 2017 http://atvb.ahajournals.org/ Downloaded from Lysophospholipids are also produced by a heterogeneous group of enzymes with PLA2 activity. Since PC is the most abundant phospholipid in cellular membranes, LPC would be the dominant lysophospholipid formed. This may have far-reaching implications for a variety of immunological/inflammatory diseases, like rheumatoid arthritis and SLE, which are characterized by an elevated PLA2 expression and activity. Our finding of antibodies to this ubiquitous lysophospholipid in healthy humans may reflect the circumstance that several types of PLA2s are constitutively expressed in many tissues and leukocytes at a low level. Several inflammatory cytokines have been shown to increase the activity and expression of PLA2s, whereas glucocorticoids (dexamethasone) inhibit the enzyme.

**TABLE 2.** Correlation Between Antibodies Against LPC, LDL, oxLDL, and CL in 210 Healthy Individuals

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Anti-LPC/ Anti-LDL</th>
<th>Anti-LPC/ Anti-oxLDL</th>
<th>Anti-LPC/ Anti-CL</th>
<th>Anti-LDL/ Anti-oxLDL</th>
<th>Anti-LDL/ Anti-CL</th>
<th>Anti-CL/ Anti-oxLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG r</td>
<td>.054</td>
<td>.281</td>
<td>.161</td>
<td>.142</td>
<td>.031</td>
<td>.03</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>&lt;.0001</td>
<td>.026</td>
<td>.0414</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>IgM r</td>
<td>.16</td>
<td>.273</td>
<td>.305</td>
<td>.864</td>
<td>.13</td>
<td>.145</td>
</tr>
<tr>
<td>P</td>
<td>.02216</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>.0394</td>
</tr>
</tbody>
</table>
Phospholipids that have previously been demonstrated to function as antigens are CL, phosphatidylethanolamine, and phosphatidylserine. Elevated levels of such antibodies are found in patients with the anti-phospholipid syndrome and are common in SLE, where they are related to enhanced risk of cardiovascular disease. It is generally believed that these phospholipids form a complex with peptides or proteins, whereby a neoantigen may be created that can be recognized by B cells. A recently published study investigating the nature of anti-CL antibodies in high-titer sera by Hörkkö et al. presented evidence that antibodies were directed against epitopes created as a result of oxidation of fatty acids in CL during overnight adherence of the lipid to the ELISA plates. The antigenicity of LPC demonstrated herein was not investigated in high-titer sera but in sera from healthy individuals. LPC-antigenicity is not likely to depend on oxidative reactions, since there was no significant difference between antibody titers to LPC and the completely saturated LPC 16:0. Furthermore, antibody titers against PC, which has two fatty acids of which one is usually unsaturated, were lower than titers against LPC and the completely saturated LPC 16:0. However, LPC and oxidized CL may share antigenic epitopes in high titer sera, a possibility presently under investigation.

We found a strong correlation between antibody titers against LPC and CL, indicating that the antigenicity of CL may also be related to hydrolysis of one or more of its four fatty acids. Thus, antigenicity in our case seems to depend on the special structure of LPC with an empty sn-2 position. Whether this structure per se is sufficient to elicit an antibody response or whether a protein-LPC complex is needed remains to be shown. It has recently been suggested that C-reactive protein binds to LPC in membranes of injured cells, thereby activating the complement cascade. Thus, C-reactive protein, which is elevated in the blood in inflammatory conditions, is a potential candidate for an LPC-complexing protein, a possibility presently under investigation.

This study confirms findings by other investigators that antibodies against oxLDL are present in normal, healthy individuals. Antibodies to native LDL were also detected but at much lower concentrations, and it is thus clear that a B-cell antigen is formed or enhanced during oxidation of LDL. We have observed that LDL often has similar effects on T-cell and monocyte/macrophage activation as does oxLDL, but that these effects in general are lower and that higher concentrations of LDL are needed. The most likely explanation is that LDL and oxLDL contain the same antigenic compound(s) but at different concentrations and that this may be formed during oxidation. Large amounts of LPC are produced during in vitro oxidation of LDL. We have demonstrated here that LPC is as effective as oxLDL in inhibiting serum binding to oxLDL. Furthermore, highly significant statistical correlations between antibody titers against native oxLDL and against LPC were found. To exclude the possibility that LPC has a capacity to inhibit antibody interaction with antigen in general, an unrelated and common antigen from Epstein-Barr virus, EB-Na, was used. In contrast to EB-Na itself, neither LPC nor oxLDL interfered with serum binding to EB-Na. Likewise, EB-Na had no capacity to inhibit serum binding to LPC or oxLDL. Furthermore, LPC inhibited binding to LPC. Taken together, our findings show that a significant part of the antigenicity of oxLDL may be explained by LPC.

Our findings may be relevant to all disorders characterized by elevated production of LPC by PLA₂ or by oxidation, because
LPC may mediate several of the proinflammatory effects ascribed to oxLDL. Other reports support the notion of LPC as a proinflammatory factor in atherogenesis, LPC potentiates protein kinase C-mediated T-cell activation, stimulates smooth muscle cell proliferation, activates protein kinase C in intact vascular segments leading to increased superoxide production, and induces monocyte mRNA expression of heparin-binding endothelial growth factor. Furthermore, LPC has been shown to be chemotactic for T lymphocytes and monocytes. An interesting possibility is that antibodies to LPC/oxLDL could form immune complexes, which could be taken up by phagocytosis in the artery wall, leading to foam cell formation. In line with this, PLA2-modified LDL is taken up by macrophages, which develop into foam cells. In principle, antibodies to LPC produced by PLA2 in LDL may therefore form immune complexes, leading to both endothelial activation and possibly foam cell formation. PLA2 and its product, LPC, free or bound to LDL, could thus be part of a comprehensive systemic as well as localized defense system against microorganisms and injured cells, which is upregulated during infection and inflammatory disorders. Clearly, studies of the role of anti-LPC antibodies in chronic inflammation and atherosclerosis could shed light on this issue.

Taken together, it is possible that LPC, produced during oxidation or enzymatically by PLA2, may activate B cells to antibody secretion and thus participate in and promote the chronic inflammatory reactions seen in atherosclerosis and several types of immunological disorders.

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