The preparation of copper-oxidized LDL for the measurement of oxidized LDL antibodies by EIA

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Received 9 June 1999; received in revised form 25 August 1999; accepted 3 November 1999

Abstract

In the present study we try to define the optimal conditions for preparation of copper-oxidized low-density lipoprotein (oxLDL) to be used for the assay of oxLDL antibodies by enzyme immunoassay (EIA). Oxidation of LDL was monitored by measuring the formation of conjugated dienes at 234 nm and the generation of fluorescent products with emission at 430 nm when excitation is performed at 360 nm. The generation of immunogenic epitopes was evaluated by testing the reactivity of aliquots collected at different times during the oxidation process with human sera with high oxLDL antibody levels and with a purified human oxLDL antibody. The values of fluorescence emission at 430 nm correlated best with reactivity with oxLDL antibodies; strong reactivity was usually associated with values greater than 1.1 U. The time needed for fluorescence emission to reach maximum levels varied between 6 and 14 h for most LDL, but it was considerably longer in a few LDL preparations. The maximal reactivity of oxLDL with oxLDL antibodies was observed when the LDL oxidation reaction was stopped 4 or more hours after the fluorescence readings reached their peak. At this stage of the oxidation reaction, apolipoprotein B fragmentation and aggregation were observed as shown by Western blot analysis. The CV for 13 EIA runs of two reference oxLDL antibodies reacting with four different pools of standardized oxLDL prepared according to the stated guidelines was 14.5 and 3.9%, confirming the reproducibility of our oxidation conditions. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Arteriosclerosis; Autoimmunity; Autoantibodies; Oxidized low-density lipoprotein antibodies; Oxidized low-density lipoprotein antibody enzyme immunoassay; Oxidized low-density lipoprotein

1. Introduction

Oxidized low-density lipoprotein (oxLDL) has been found to have an important role in the development of atherosclerosis. The pathogenic mechanisms by which oxLDL leads to arteriosclerosis have been the object of intense investigation, and remain to be fully defined. It has been clearly established that oxLDL is immunogenic, leading to the formation of antibody complexes [1–8] and immune complexes [9,10] which may have a pathogenic role. Antibodies reactive with oxLDL (anti-oxLDL) have been detected [1–10] and isolated [11] from the serum of patients with vascular disease and from the serum of apparently healthy subjects. Studies showing a correlation between increased levels of oxLDL antibodies and the presence or progression of vascular lesions [1,3–6,8] support the pathogenic role of these antibodies, as do the observations that oxLDL antibodies recognize epitopes in atherosclerotic lesions of rabbits and humans but not in normal arteries [12–14]. However, other groups have failed to demonstrate any significant correlation between the detection of oxLDL antibodies and clinical or radiological findings of atherosclerosis [2,7].

One possible explanation for these contradictory observations may be the lack of standardization of the assays used for the measurements of oxLDL antibodies by different groups. Enzyme immunoassay (EIA) is the most commonly used method for the determinations of
oxLDL antibodies [1–11]. We and others [1] have encountered problems with batch-to-batch variability in reactivity of oxLDL antibodies when copper oxidized LDL has been used as antigen in EIA. This seems to reflect variations in the degree of oxidation of the different LDL preparations used to perform the assay. One of the parameters which is usually not carefully controlled is the duration of the oxidation reaction, which is stopped after an arbitrarily defined time period. This may result in the use of insufficiently oxidized LDL preparations. However, even when the reaction is allowed to proceed to its maximum, the extent of modification varies in different LDL preparations. The most widely used method to determine the extent of LDL modification is the measurement of thiobarbituric acid reactive substances (TBARS) [15]. This method is known to have low specificity [16] and, in our experience, fails to reflect the batch-to-batch variability of oxLDL for the products (epitopes) recognized by oxLDL antibodies. Alternative methods to determine the extent of LDL modification include the measurement of lipid peroxides, conjugated dienes and aldehyde formation, all indicative of lipid peroxidation during the early phase of the oxidation process [16,17] as well as the measurement of fluorescent products with emission at 430 nm when excitation is performed at 360 nm, indicative of apolipoprotein B modification [16,18]. Thus, we decided to determine whether continuous monitoring of the oxidation of LDL by sensitive techniques would give a more precise indication about the extent of LDL modification needed to react well with serum ox-LDL antibodies and therefore allow a more rational approach to consistently prepare oxLDL for the assay. The techniques chosen for our study were the measurement of conjugated dienes and of fluorescent compounds formed during LDL oxidation. The results presented in this article show that monitoring the fluorescent compounds allows us to determine the stage and extent of LDL oxidation associated with a better recognition by oxLDL antibodies. This criterion is far superior to reaction time or to the TBARS assay to determine if and when the optimal degree of LDL oxidation has been reached, thus allowing us to prepare batches of oxLDL which exhibit consistent reactivity with oxLDL antibodies.

2. Materials and methods

2.1. Lipoprotein isolation, modification and characterization

Blood for lipoprotein isolation was collected from normal healthy volunteers in 0.4 mmol/l EDTA after 12 h of fasting. The donors used for this purpose were normolipemic healthy volunteers, not receiving prescription medication for any acute or chronic condition and without family history of coronary artery disease, peripheral vascular disease, or stroke. None of the volunteers was receiving anti-oxidant therapy. LDL was isolated from individual or pooled plasma, after density adjustment (1.019 < density < 1.063 g/ml) with potassium bromide (KBr), by preparative ultracentrifugation at 50 000 rpm for 17 h on a Beckman L-80 ultracentrifuge, using a type 70 Ti rotor [19]. LDL, isolated as described, was washed by ultracentrifugation, dialyzed against a 0.15-mol/l sodium chloride solution containing 300 μmol/l EDTA, pH 8.0, passed through an Acrodisc filter (0.22-μm pore size) in order to sterilize and remove aggregates, and stored under nitrogen in the dark at 4°C.

For oxidation, we used two individual LDL preparations and eight different LDL pools, each one of the pools containing LDL isolated from three different individuals. All pools and individual LDL preparations were oxidized shortly after blood collection, usually within 2–3 weeks and never exceeding 4 weeks after blood collection (except when otherwise noted). Of the LDL pools four were split into two aliquots. Of these aliquots four were oxidized during the first 2 weeks after blood collection; of the remaining four aliquots, two were oxidized 5–5.5 weeks after blood collection, and the remaining two were oxidized 9 weeks after blood collection.

Oxidation was performed according to the protocol described by Steinbrecher et al. [20]. To remove residual KBr and EDTA prior to starting the oxidation reaction, the LDL was passed through a PD-10 column (Pharmacia Biotech, Uppsala, Sweden). Phosphate buffered saline (PBS), pH 7.4, was oxygenated at 2 l/min for 10 min and LDL was diluted by PBS to a final concentration of 1500 mg/l of ApoB. A stock solution of copper chloride (CuCl₂, 10 mmol/l in distilled water) was added to the LDL preparation to a final concentration of 40 μmol/l. After addition of CuCl₂, the LDL preparation was filtered through a 0.22-μm filter and incubated at 37°C. In order to track the degree of oxidation, two aliquots of every LDL preparation, adjusted to an ApoB concentration of 50 mg/l, were used for continuous monitoring of fluorescence and formation of conjugated dienes. Preliminary comparisons of data generated by continuous monitoring as described above and by analysis of timed aliquots collected from the batch undergoing oxidation after dilution to 50 mg of ApoB/l showed identical results. Fluorescence was monitored at 37°C on a luminescence spectrophotometer (SLM-AMINCO® Series 2, Spectronic Instruments, Rochester, NY). The samples were placed on covered quartz cuvettes; a wavelength of 360 nm was set for excitation and a wavelength of 430 nm was set to measure fluorescence emission [16,18]. The slit was set to a 4-nm bandpass.
The monitored samples were not stirred, to more closely reproduce the conditions of oxidation of the experimental batches. The values were expressed as fluorescence units, using a 0.1-μg/ml quinine solution to adjust the sensitivity of the fluorometer (the gain was adjusted to obtain a reading of 2.6 U with the quinine solution). Conjugated dienes were measured on a Beckman spectrophotometer (model DU 640 with temperature control) at 234 nm [16,17]. The temperature was set at 37°C and the cells were not stirred.

Small aliquots of the main LDL preparation were harvested under sterile conditions at different stages of the oxidation reaction. The oxidation in each collected sample was stopped by the addition of EDTA and butyl-hydroxytoluene (BHT) to a final concentration of 0.3 and 0.2 mmol/l, respectively. Copper was then removed from the oxLDL samples by overnight dialysis against 0.15 mol/l NaCl containing 0.3 mmol/l EDTA, pH 8.0. After dialysis the oxLDL samples were filtered through a 0.22-μm filter to remove large aggregates. The final protein concentration was determined after filtration by a Lowry assay [21]. Recovery data was consistently at ~80% of the initial protein concentration for samples oxidized for periods not exceeding 24 h. Protein recovery in samples oxidized for longer periods of time was slightly lower, usually not below 70% of the initial protein concentration.

The degree of LDL denaturation associated with copper oxidation was assessed by two separate criteria: Western blot analysis and agarose gel electrophoresis. For both assays we used samples collected at different oxidation times. For Western blot, the samples were dissolved in a buffer containing 63 mmol/l Tris–HCl, 2% SDS, 10% glycerol, 0.01 mmol/l BHT, and 0.001% bromophenol blue (pH 6.8), heated for 3 min in a boiling water bath, and then run for 2.5 h, at 100 V in a 4-20% PAGE gel in 25 mmol/l Tris glycine buffer, pH 8.3, containing 0.1% SDS (Novex, San Diego, CA). After electrophoresis, the proteins were electrotransferred to PVDF membranes in 15 mmol/l Tris-glycine buffer, pH 8.3, with 20% (v/v) methanol. Membranes were blocked with 5% fat-free dry milk for 1 h at room temperature, and then incubated for another hour with a 1:30 000 dilution of rabbit antiserum to human apolipoprotein B (Behring, Somerville, NJ) in TBS-T-5% milk solution. After incubation with this antiserum the membranes were washed five times with TBS-T and then incubated for 1 h with a 1:20 000 dilution of sheep anti-rabbit IgG HRP conjugated antibody in the same buffer. Afterwards the membranes were washed five times with TBS-T and the immunoreactive proteins were visualized by treating the membranes with ECL detection reagents (Amersham, Arlington Heights, IL) followed by exposure of Kodak X-Omat film.

Agarose gel electrophoresis of native and modified LDL was performed in 0.5% (w/v) gel, prepared in 0.05 M sodium barbital buffer, pH 8.6 and subjected to 25-mA constant current for 2.5 h at 4°C. The gels were fixed for 1 h in 5% glacial acetic acid, 75% ethanol (v/v) and air dried. LDL bands were visualized after staining the gel in a saturated solution of oil red O (0.04% in 60% ethanol, stored at 4°C).

2.2. Human oxLDL antibodies

To test the reactivity of the various preparations of copper-oxidized LDL prepared as described above, six human sera with concentrations of oxLDL antibody ranging from 20 to 204 mg/l determined by enzyme immunoassay were used. A purified human oxLDL antibody with a total antibody concentration of 88 mg/l was also used to test the reactivity of some oxLDL batches. This oxLDL antibody was isolated from human serum by affinity chromatography as described previously [11,22], had a dissociation constant (Kd) of 6.6 × 10⁻⁹ mol/l, and was constituted predominantly by IgG (68.5%). None of the reference antibodies was obtained from the same individuals who donated plasma for LDL isolation. Previous studies have shown that isolated oxLDL antibodies have marked cross-reactivity with MDA-LDL, but react very poorly with glycated LDL, native LDL, oxidized human serum albumin, or cardiolipin [11].

2.3. Enzyme immunoassay

The reactivity of different oxLDL preparations with oxLDL antibodies was tested by a competitive enzyme immunoassay (EIA) we have previously described [2,22]. The assay was conducted on flat-bottom Immulon type 1 EIA plates (Dynatech, Chantilly, VA). The wells were coated by overnight incubation at 4°C with 100 μl of oxLDL (7.5 mg/l LDL in 1 mol/l carbonate-bicarbonate buffer, pH 9.6). Serum samples prediluted 1/10 in PBS containing 1% bovine serum albumin (BSA, Cohn Fraction V- cat. # A-7906, St Louis, MO, USA) (PBS-BSA) and isolated antibody were separated into two 200-μl aliquots. One of the aliquots was absorbed with an equal volume of oxLDL (400 μg/ml in PBS-BSA) and the other (unabsorbed) was mixed with an equal volume of PBS-BSA. The final dilution of all serum samples was 1/20. All samples (absorbed and unabsorbed) were incubated overnight at 4°C on a rocker. After the incubation, the plates were blocked with 5% BSA in PBS (1 h at room temperature) and then

Fig. 1. Results of a 24-h continuous monitoring during copper oxidation of a LDL pool of conjugate diene formation, measured by spectrophotometry at 234 nm (left panel) and of ApoB-emitted fluorescence, determined by exciting the LDL solution at 360 nm and measuring emitted fluorescence at 430 nm (right panel). The arrows and letters indicate the aliquots harvested for testing their reactivity with reference sera with known concentrations of oxLDL antibodies (Fig. 2) and LDL denaturation by Western blot and agarose gel electrophoresis (Fig. 3).

washed four times with 0.05% Tween 20 (Sigma) in PBS, pH 7.8. The absorbed and unabsorbed aliquots were spun at 9000 × g in an Eppendorf centrifuge (model 5413) for 30 min. The supernatants of the centrifuged samples, unabsorbed and absorbed, were tested at two final dilutions: 1:2 and 1:4 for purified antibodies and 1:20 and 1:40 for sera. Then 100 µl of both dilutions were transferred to the wells of the oxLDL-coated plates and after overnight incubation at 4°C and washing, 150 µl of peroxidase-conjugated rabbit anti-human IgG (IgG fraction) (Cappel Organon Teknika, Durham, NC), diluted 1:5000 in PBS-BSA, was added to each well. After incubation for 1 h at 4°C, the unbound conjugate antibody was washed off, and 0.5 mmol/l of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS, Sigma, St Louis, MO) and 3% (v/v) hydrogen peroxide in a 45-mmol/l concentration of citric acid buffer, pH 4.0, was added as substrate. Color was allowed to develop for 10 min at room temperature, in the dark. The reaction was stopped with 0.1 mol/l citric acid, pH 2.1, and the absorbance was measured at 414 nm in a VMax enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, CA). The optical density values were calculated by subtracting the OD values measured on oxLDL-absorbed aliquots from those measured on unabsorbed aliquots at identical dilutions. A human oxLDL antibody standard was used to calculate the concentration of oxLDL antibody in serum and isolated antibody preparations as described in an earlier publication [22].

In all EIA studies testing different oxLDL preparations the same oxLDL preparation was used to coat the wells of the Immulon plates and to absorb free antibody on antibody-containing samples.

3. Results

The longitudinal changes in the formation of fluorescent compounds and of conjugated dienes during oxidation of a LDL pool (pool 1) for 24 h are presented in Fig. 1. The nine aliquots of this LDL pool (labeled ‘a’ to ‘i’ in the figure) were obtained at different stages of oxidation and tested for their reactivity with five different reference sera (Fig. 2). In the example presented in Fig. 1, fluorescence emission at 430 nm reached its maximum level ~6–7 h after starting oxidation. We observed maximal reactivity with oxLDL antibodies 4 h

Fig. 2. Study of the reactivity of a series of aliquots of LDL harvested at different times during the oxidation reaction (Fig. 1) with five different reference oxLDL antibodies. The antibody concentration in the five reference sera was 47 mg/l (serum 1), 45 mg/l (serum 2), 20 mg/l (serum 3), 27 mg/l (serum 4), 204 mg/l (serum 5). The results are expressed as the difference in OD measured in unabsorbed and absorbed aliquots of each one of the sera, using identical LDL samples for EIA plate coating and antibody absorption.
Fig. 3. Top panel: Reproduction of a Western blot analysis of the degree of LDL fragmentation and aggregation associated with different times of oxidation. Native LDL (a) and five samples collected at the times shown in Fig. 1 were separated by SDS–PAGE and exposed to a polyclonal rabbit human ApoB antibody. Samples 'g' and 'i' had maximal reactivity with reference oxLDL antibodies (Fig. 2). The positions of molecular weight standards included in the run are indicated on the left margin of the figure. Bottom panel: Reproduction of an agarose gel electrophoretogram of the samples separated by Western blot. The samples were applied at origin (O).

The wide range of time required to reach maximal modification was even more dramatically illustrated with an LDL pool that showed increasing fluorescence values for over 24 h of oxidation (Table 1). In that particular study monitoring was done at various time periods and the highest values of fluorescence were recorded in aliquots harvested after 48 h of oxidation, which also showed maximum reactivity with reference oxLDL antibodies. Both parameters remained stable when oxidation of LDL was continued for an additional 24 h after peak fluorescence was reached.

The experiments described above suggest that once the maximum levels of fluorescence and antibody reac-

<table>
<thead>
<tr>
<th>Time of oxidation (h)</th>
<th>2</th>
<th>10</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence (360–430 nm)</td>
<td>0.440</td>
<td>1.680</td>
<td>1.800</td>
<td>2.600</td>
<td>3.801</td>
<td>3.930</td>
</tr>
<tr>
<td>Reactivity with purified antibody</td>
<td>0.106</td>
<td>0.253</td>
<td>0.242</td>
<td>0.350</td>
<td>0.696</td>
<td>0.695</td>
</tr>
<tr>
<td>Reactivity with reference serum</td>
<td>0.024</td>
<td>0.065</td>
<td>0.067</td>
<td>0.158</td>
<td>0.342</td>
<td>0.361</td>
</tr>
</tbody>
</table>

* Antibody reactivity values are expressed as optical density values calculated by subtracting the OD measured at 414 nm on oxLDL absorbed-aliquots from the OD measured on unabsorbed aliquots of our reference oxLDL antibody and of a reference serum containing 201 mg/l of oxLDL antibody. The EIA wells were coated with LDL samples collected during oxidation at the indicated times.
Reactivity of oxLDL aliquots of an LDL pool harvested at 3, 17, 36, and 47 h, after the fluorescence reached the maximum level with purified oxLDL antibody, and two reference sera containing 47 mg/l of oxLDL antibody (serum 1) and 204 mg/l of oxLDL antibody (serum 5) 

<table>
<thead>
<tr>
<th>Antibody</th>
<th>3 h</th>
<th>17 h</th>
<th>36 h</th>
<th>47 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified Ab</td>
<td>0.576</td>
<td>0.774</td>
<td>0.755</td>
<td>0.789</td>
</tr>
<tr>
<td>Serum 1</td>
<td>0.208</td>
<td>0.424</td>
<td>0.460</td>
<td>0.466</td>
</tr>
<tr>
<td>Serum 5</td>
<td>0.478</td>
<td>0.608</td>
<td>0.543</td>
<td>0.551</td>
</tr>
</tbody>
</table>

Reactivity is expressed as optical density values calculated by subtracting the OD values obtained on oxLDL-absorbed aliquots from those measured on unabsorbed aliquots. Final dilutions were 1/20 for serum and 1/2 for isolate.

Table 2

Reactivity of oxLDL aliquots of an LDL pool harvested at 3, 17, 36, and 47 h, after the fluorescence reached the maximum level with purified oxLDL antibody, and two reference sera containing 47 mg/l of oxLDL antibody (serum 1) and 204 mg/l of oxLDL antibody (serum 5) 

<table>
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Reactivity is expressed as optical density values calculated by subtracting the OD values obtained on oxLDL-absorbed aliquots from those measured on unabsorbed aliquots. Final dilutions were 1/20 for serum and 1/2 for isolate.

Table 3

Study of the effect of LDL storage prior to oxidation on the levels of fluorescence and conjugated dienes measured after oxidation

<table>
<thead>
<tr>
<th>LDL pool no.</th>
<th>Fluorescence</th>
<th>Conjugated dienes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time to reach the maximal level of fluorescence (h)</td>
<td>Peak fluorescence value (360–430 nm)</td>
</tr>
<tr>
<td></td>
<td>Fresh LDLb</td>
<td>Stored LDLc</td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>13</td>
<td>8</td>
</tr>
</tbody>
</table>

One half of four LDL pools was oxidized within 4 weeks of isolation of the different LDL fractions included in the pool (fresh LDL) and the other half was oxidized after 5–9 weeks of storage (stored LDL). The time of oxidation needed to reach the maximum level of fluorescence and the corresponding values for fluorescence, as well as the levels of conjugated dienes after 12 h of oxidation are presented in the table.

a One half of four LDL pools was oxidized within 4 weeks of isolation of the different LDL fractions included in the pool (fresh LDL) and the other half was oxidized after 5–9 weeks of storage (stored LDL). The time of oxidation needed to reach the maximum level of fluorescence and the corresponding values for fluorescence, as well as the levels of conjugated dienes after 12 h of oxidation are presented in the table.

b Oxidized within 2 weeks of blood collection.

c Pool 5 was oxidized 5 weeks after blood collection; pool 6 was oxidized 5.5 weeks after blood collection; pools 7 and 8 were oxidized 9 weeks after blood collection.
Table 4
Study of the effect of LDL storage prior to oxidation on the reactivity with reference oxLDL antibodies

<table>
<thead>
<tr>
<th>LDL pool no.</th>
<th>Fresh LDL</th>
<th>Stored LDL</th>
<th>Reactivity with reference serum 1</th>
<th>Reactivity with reference serum 5</th>
<th>Reactivity with reference serum 1</th>
<th>Reactivity with reference serum 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fresh LDL</td>
<td>Stored LDL</td>
<td>Fresh LDL</td>
<td>Stored LDL</td>
</tr>
<tr>
<td>5</td>
<td>0.47</td>
<td>0.36</td>
<td>0.76</td>
<td>0.63</td>
<td>0.46</td>
<td>0.85</td>
</tr>
<tr>
<td>6</td>
<td>0.52</td>
<td>0.35</td>
<td>0.84</td>
<td>0.62</td>
<td>0.54</td>
<td>0.84</td>
</tr>
<tr>
<td>7</td>
<td>0.54</td>
<td>0.36</td>
<td>0.84</td>
<td>0.69</td>
<td>0.52</td>
<td>0.76</td>
</tr>
<tr>
<td>8</td>
<td>0.52</td>
<td>0.35</td>
<td>0.84</td>
<td>0.62</td>
<td>0.54</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* The processing of LDL pools was as detailed in Table 3. Reactivity is expressed as optical density values calculated by subtracting the OD_{414} values corresponding to the reactivity of a given oxLDL pool with an unabsorbed aliquot of a reference oxLDL-containing sera and the OD_{414} corresponding to the reactivity of the same oxLDL pool with a pre-absorbed aliquot of the same reference serum.

b Oxidized within 2 weeks of blood collection

c Pool 5 was oxidized 5 weeks after blood collection; pool 6 was oxidized 5.5 weeks after blood collection; pools 7 and 8 were oxidized 9 weeks after blood collection.

pools of oxLDL were used as antigens. Fig. 4 reproduces graphically the values (mean ± 1 S.D.) obtained with these two quality control samples; the coefficients of variation calculated from these values were of 14.4% for the low control and 3.9% for the high control. These CV compared favorably with between-run CV calculated with the same samples using a single LDL pool (7.4 and 3.0%, respectively; n = 5).

4. Discussion

The lack of consistency in the results of anti-oxLDL surveys published by different groups [1–11] has strongly suggested that at least some of the contradictions between different studies may result from the lack of standardization of the assays currently used for measurement of oxLDL antibodies. We and others [1] have used copper oxidized LDL as antigen in the antibody assay, because of its similar characteristics to the LDL isolated from human atheromatous plaques [5–9]. The protocols used by different groups for preparation of copper oxidized LDL have in common the fact that the oxidation reaction is usually stopped after a given period of time, usually between 18 and 24 h [3,5–8,11]. The degree of oxidation has been assessed most often by measurements of TBARS [15] and, less frequently, of conjugated dienes [3,16,23,24]. Since the lack of sensitivity and accuracy of the measurements of TBARS is well known [16], we decided to measure conjugated dienes to monitor the formation of lipid hydroperoxides during LDL oxidation, and to measure fluorescence to monitor the modification of ApoB and the formation of protein/aldihyde adducts. We then studied the correlation between these parameters and the reactivity with reference sera with known oxLDL antibody contents and with an oxLDL antibody isolated by affinity chromatography.

The formation of conjugated dienes can be measured spectrophotometrically by monitoring absorbance at a wavelength of 234 nm. To monitor the degree of apo-protein oxidation we relied on the measurement of fluorescent products generated as a consequence of conjugation of aldehyde fragments released by oxidized fatty acids to amino groups [18]. These fluorescent compounds show a maximum emission at 430 nm when excitation is performed at 360 nm.

Since the immunoreactivity of oxLDL is dependent on the modification of ApoB, it was hoped that the fluorescence values would closely reflect the structural changes of ApoB that are associated with immunogenicity. This postulate was supported by our findings, showing that fluorescence monitoring gives reliable information on the degree of modification of LDL and that purified oxLDL antibody reacts most avidly with LDL after the formation of protein/aldihyde adducts reaches maximum levels. Maximal reactivity with reference oxLDL antibodies is also associated with elec-

![Fig. 4. Diagrammatic representation of the mean ± 1 S.D. of the concentrations of oxLDL antibody measured with two reference sera (1 and 5) over a 5-month period, using four different oxLDL preparations as antigens. The antibody concentration for serum 5 was 204 ± 8 mg/l and for serum 1 was 47 ± 7 mg/l.](image-url)
tion of the LDL fatty acids. Either change could cause prolonged LDL storage induces either a mild degree of weeks after blood collection with reference sera was the reactivity of LDL aliquots oxidized more than 4 for those oxidized shortly after LDL isolation. Also, and the peak values were lower for LDL aliquots showed that peak values were reached in shorter times, Monitoring of fluorescence and conjugated dienes prior to oxidation we observed inconsistent results. The distinct and early peak of conjugated dienes observed at 234 nm during the oxidation reaction may permit estimation of the optimal oxidation time, since it usually precedes the fluorescence peak by 4–6 h. However, there is a poor correlation between the levels of fluorescence and the levels of conjugated dienes in the same LDL preparations, and just monitoring the conjugate diene peak would not allow determination of the extent of fluorescence.

Our observations underscored the need to stop the oxidation reaction when it is complete, rather than at a fixed time. On the other hand, the reactivity of oxLDL with defined antibodies seems to become stable 4–6 h after the fluorescence values reached their peak. In one experiment in which an LDL pool was oxidized for a total of 56 h, antibody reactivity remained stable even when LDL was collected 47 h after the maximum level of fluorescence was reached (Table 1). However, to avoid possible interference of excessive LDL fragmentation, which is known to be more pronounced after long periods of oxidation, we prefer to stop oxidation within 4–6 h after fluorescence has reached its peak.

The stability of the immunodominant epitopes of oxLDL during the oxidative process was greater than expected. However, when we tried to oxidize LDL aliquots that had been stored for more than 4 weeks prior to oxidation we observed inconsistent results. Monitoring of fluorescence and conjugated dienes showed that peak values were reached in shorter times, and the peak values were lower for LDL aliquots oxidized more than 4 weeks after blood collection than for those oxidized shortly after LDL isolation. Also, the reactivity of LDL aliquots oxidized more than 4 weeks after blood collection with reference sera was lower than the reactivity of LDL aliquots oxidized soon after LDL isolation. Thus, it appears probable that prolonged LDL storage induces either a mild degree of LDL delipidation or a change in the degree of saturation of the LDL fatty acids. Either change could cause not only a reduction in the generation of conjugated dienes during copper oxidation but also in the formation of fluorescent compounds and, consequently, a relative paucity of the epitopes recognized by oxLDL antibodies. This has led us to establish that for preparation of oxLDL to be used in antibody assays we should use LDL pools stored for as short a time as possible, never exceeding 4 weeks after blood collection.

A crucial test for the validity of our protocol for oxLDL preparation was to verify that the reactivity of reference oxLDL antibodies would remain constant when different oxLDL preparations were used. This was confirmed using data from a total of 13 separate runs in which four different oxLDL pools were used as antigens and the same two quality control samples were included in the runs (Fig. 4). The calculated concentrations for the quality control samples remained very stable, and the coefficients of variation calculated with the data generated in those 13 runs compared favorably with between run CV determined for the same quality controls using a single batch of oxLDL. Thus, when oxLDL is prepared from a pool of freshly isolated LDL and the oxidation reaction is stopped 4–6 h after a maximum fluorescence value is reached and the fluorescence value is greater than 1.1 U, the resulting oxLDL preparations are remarkably homogeneous in their reactivity with oxLDL antibodies. The use of oxLDL prepared according to these principles has resulted in increased consistency of the data generated in our oxLDL antibody assay [2], as reflected by closer agreement of the values for the quality control samples included in each run.

In conclusion, by carefully defining the conditions for preparation of copper-oxidized LDL and by carefully monitoring the oxidation reaction we have been able to obtain oxLDL preparations that exhibit consistent behavior in our oxLDL antibody assay. Monitoring fluorescence as the oxidation reaction progresses allows us to determine when the reaction should be stopped for individual LDL pools and, more importantly, allows us to discard insufficiently modified LDL preparations. Such preparations do not behave consistently in the oxLDL antibody assay and would otherwise not be identified until the results of the oxLDL antibody enzymoimmunoassay became available.

Acknowledgements

This work was supported by the National Institutes of Health grant HL-55782, by the Research Service of the Department of Veterans Affairs, and by grants from the Finnish Foundation of Diabetes Research, the Medical Council of the Academy of Finland, the Po-hjois-Karjala Cultural Foundation, the Aarne Koskelo Foundation, and the Jalmari and Rauha Ahokas Foun-
dation (Dr S. Koskinen). Dr Sinikka Koskinen is a post-doctoral fellow of the Juvenile Diabetes Foundation International. The authors wish to thank Gregor Krings for technical assistance and Rebecca Rollins for editorial assistance.

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