ELISA of IgG Antibody to Oxidized Low-Density Lipoprotein: Effects of Blocking Buffer and Method of Data Expression

Wendy Y. Craig,1 Sue E. Poulin, Caleb P. Nelson, and Robert F. Ritchie

We describe an ELISA for serum IgG antibodies against malondialdehyde-modified low-density lipoprotein (mLDL). Optimal antigen concentration, serum dilution, and dilution of enzyme-conjugated second antibody were 25 mg/L, 1:250, and 1:5000, respectively, when 5 g/L human serum albumin was used for blocking. When data were expressed as mLDL/LDL (the ratio of IgG binding to mLDL vs LDL), within-run and between-run CVs were 7.0% and 8.9%, respectively. Antibody concentrations expressed as mLDL/LDL or as mLDL – LDL (the difference between IgG binding to mLDL and to LDL) were higher in women with systemic lupus erythematosus (n = 20) than in controls (n = 20) (P <0.001). With bovine serum albumin or Superblock™ blocking buffers, only the mLDL – LDL data were significant. Thus, the choice of blocking agent and the method of data expression should be carefully considered when assaying IgG antibodies against mLDL.

Indexing Terms: atherosclerosis/cholesterol/systemic lupus erythematosus/autoantibodies/variation, source of

There is considerable interest in the relation between oxidative processes and the development of atherosclerosis (1). Low-density lipoprotein (LDL) that has been modified, e.g., by oxidation, is capable of loading macrophages with cholesterol, whereas native (unmodified) LDL is not (2); thus, oxidized LDL is considered particularly atherogenic.2 Concentrations of thiobarbituric acid-reactive substances (TBARS), a nonspecific measure of lipid peroxidation, are greater in subjects with coronary artery disease than in controls (3), and increased amounts of IgG autoantibody against oxidized LDL are associated with the progression of carotid atherosclerosis (4).

Evaluation of lipoprotein oxidation in vitro is difficult, given the multiple chemical effects of oxidation; extreme care must be taken to avoid degradation of the sample. Also, the correlation between concentrations of oxidation products in the circulation and events at the atherosclerotic plaque (which may be the primary site of oxidation) is uncertain. Chait (5) suggested that measuring autoantibodies against oxidized LDL might be useful for evaluating LDL oxidation.

Serum IgG autoantibodies against modified LDL have been reported by several groups using ELISA (6, 7) or RIA (8) methods and have been quantified in terms of titers (8), the mean absorbance of wells containing 12 doubling dilutions of serum (6), the ratio of IgG binding to malondialdehyde-modified LDL (mLDL) and to LDL (mLDL/LDL) (4), the difference in absorbance readings between IgG binding to mLDL and to LDL (mLDL–LDL) (9), and the difference in binding of IgG to oxidized LDL between untreated serum and serum preabsorbed with oxidized LDL (7). Other immunoglobulin classes may, however, also be important (10).

Here, we describe in detail the development of an ELISA to quantify IgG autoantibodies against mLDL, using the ELISA to confirm the results of Vaarala et al. (9)—that serum concentrations of IgG autoantibodies against mLDL are increased in subjects with systemic lupus erythematosus (SLE)—with sera from 20 females with SLE and 20 age-matched non-SLE controls, and to examine the effects of different blocking buffers and methods of data expression on experimental findings.

Materials and Methods

Reagents

MaxiSorp F96 microtiter plates were from Nunc (Kamstrup, Denmark). Superblock™ blocking buffer in phosphate-buffered saline (PBS) was a gift from Pierce (Rockford, IL). Aprotinin, chloramphenicol, benzamidine, phenylmethylsulfonyl fluoride, o-phenylenediamine dihydrochloride, butylated hydroxytoluene, citric acid, potassium bromide, human serum albumin Fraction V (HSA), bovine serum albumin Fraction V (BSA), 2-thiobarbituric acid, gentamicin, and 1,1,3,3-tetramethoxypropane were from Sigma Chemical Co. (St. Louis, MO). Sodium azide was from E. M. Science (Gibbstown, NJ); EDTA, from Fisher (Fair Lawn, NJ); trichloroacetic acid, from J. T. Baker (Phillipsburg, NJ); horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG Fc fragment (affinity purified, lot no. 14588, 1.0 g/L antibody), from Jackson ImmunoResearch Labs (West-grove, PA); and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone, from Chemica Alta (Edmonton, Cana-da). All other chemicals were reagent grade.

Sera

The assay was developed with sera received for unrelated clinical testing in our laboratory; these sera were stored in aliquots for 6 months to 2 years at –20°C before use. Sera from subjects with and without SLE were identified from samples that entered our labora-

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1 Address correspondence to this author. Fax 207-883-1527.
2 Nonstandard Abbreviations: LDL, low-density lipoprotein; mLDL, malondialdehyde-modified low-density lipoprotein; SLE, systemic lupus erythematosus; HSA, human serum albumin; BSA, bovine serum albumin; mLDL/LDL, ratio of IgG binding to mLDL and to LDL; mLDL–LDL, difference between IgG binding (A₄₅₀) to mLDL and to LDL; TBARS, thiobarbituric acid-reactive substances; HRP, horseradish peroxidase; and PBS, phosphate-buffered saline.

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LDL isolation and modification. Blood was drawn into tubes containing EDTA, and preservatives were added to the separated plasma to give the following final concentrations, per liter: gentamicin, 100 mg; chloramphenicol, 0.15 mmol; phenylmethylsulfonyl fluoride, 1 mmol; EDTA, 2.7 mmol; benzamidine, 2 mmol; apro- tinin, 0.1 g; and D-phenylalanil-L-prolyl-L-arginine chloromethyl ketone, 1 mmol. We isolated LDL by sequential ultracentrifugation (11), using PBS (10 mmol/L sodium phosphate, 0.15 mol/L NaCl, pH 7.4) containing the above preservatives to overlay the plasma. The LDL (density 1.019–1.063 g/L) fraction was further purified by a second centrifugation. After isolation, LDL was dialyzed against PBS containing 100 mg of gentamicin, 1 mmol of phenylmethylsulfonyl fluoride, and 0.1 mmol of EDTA per liter. LDL protein content was assayed according to Lowry et al. (12). The LDL preparation was then divided into two parts: half kept as native LDL and half modified by conjugation with malondialdehyde, as described by Palinski et al. (13). After the conjugation reaction, mLDL was dialyzed overnight at 4°C against dialysis buffer (above). We then assayed the malondialdehyde content of native and mLDL as TBARS, as described by Lamb and Leake (14), using tetramethoxypropane standards as described by Wong et al. (15). Both native and MDA-conjugated LDL preparations were stored at 4°C for as long as 3 months.

ELISA of IgG antibody against mLDL. The method was developed as a modification of the RIA procedure of Palinski et al. (13). The exact experimental conditions (antigen concentration, blocking buffer composition, serum, and enzyme-conjugated second antibody dilutions) used in specific experiments are given below; optimized conditions are presented in Table 1.

Preparations of LDL or mLDL (both diluted in antigen buffer: PBS containing 2.7 mmol/L EDTA and 20 μmol/L butylated hydroxytoluene), or buffer alone (antigen blank), were loaded (50 μL each) in three-lane series across a microtiter plate for all optimization experiments; for routine analysis of sera, alternate lanes were loaded with LDL and mLDL (no antigen blank). After incubation for 20 h at 4°C, the antigen solutions were aspirated and the plates were washed three times with 200 μL (quick wash) and once again with 200 μL (10–15 min) of wash buffer (PBS containing 3.08 mmol/L sodium azide, 10 mg/L aprotinin, and 0.5 mL Tween 20, 4°C). The antigen-coated plates were used immediately after preparation. Wells were blocked by incubation at room temperature for 45 min with 250 μL/well of blocking buffer and were washed as described above before incubation at 4°C for 20 h with serum, 50 μL/well, diluted either in blocking buffer (when BSA or Superblock was used for blocking) or in PBS diluent (PBS containing 10 mg/L aprotinin and 2.7 mmol/L EDTA) (when HSA was used for blocking). The plates were then aspirated and washed as above, and incubated at 4°C for 4 h with HRP-conjugated rabbit antihuman IgG, 100 μL/well, diluted either in blocking buffer or in PBS diluent, as appropriate. After washing, color was developed by incubating the plates in the dark for 6 min 40 s with 100 μL/well of substrate (15 mg of o-phenylenediamine, 28.4 μL of 30% hydrogen peroxide in 60 mL of 58 mmol/L disodium phosphate, and 13.7 mmol/L citric acid, pH 5.6). The reaction was stopped with 2.5 mol/L sulfuric acid, 50 μL/well, and A490 was measured by an EL309 microplate reader from Biotek Instruments (Winooski, VT).

Expression of data. The concentrations of serum IgG antibodies against mLDL were expressed either as mLDL/LDL, mLDL–LDL, or as normalized mLDL–LDL (to take into account variations in absorbance between runs). To normalize the absorbance data, we diluted 1:50-fold a serum pool (n = 3 sera) that had been stored in aliquots at −20°C and determined its antibody binding to mLDL in quadruplicate on each ELISA plate. The difference in absorbance between antibody binding to mLDL and to LDL for each serum sample was then expressed as

<table>
<thead>
<tr>
<th>Blocking buffer</th>
<th>Wash buffer, conc per L of PBS</th>
<th>Diluent*</th>
<th>Antigen conc, mg/L</th>
<th>Enzyme-conj. 2nd Abb</th>
<th>Serum conc, mg/L</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 g/L BSA in PBS</td>
<td>0.54 mmol NaB, 10 g aprotinin, 0.5 mL Tween 20</td>
<td>Superblock</td>
<td>5.4 mmol NaB, 10 g aprotinin, 0.5 mL Tween 20</td>
<td>10 mg/L HSA in PBS</td>
<td>1:100</td>
<td>1:10 000</td>
</tr>
<tr>
<td>5 g/L HSA in PBS</td>
<td>0.54 mmol NaB, 10 g aprotinin, 0.5 mL Tween 20</td>
<td>Superblock</td>
<td>5.4 mmol NaB, 10 g aprotinin, 0.5 mL Tween 20</td>
<td>10 mg/L HSA in PBS</td>
<td>1:250</td>
<td>1:5 000</td>
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</table>

*a For both serum and enzyme-conjugated second antibody.

b Enzyme-conjugated second antibody concentration (undiluted) was 1.0 g/L.

Table 1. Optimal assay conditions.

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a percentage of the absorbance of the serum pool binding to mLDL.

Anti-phospholipid antibody assays. Anti-cardiolipin and anti-phosphatidyl serine antibodies were assayed by the method of Gharavi et al. (16).

Mathematical methods. Linear regression was used to examine (a) the relation between the assayed antibody concentrations and the age of the antigen (LDL, mLDL) preparations, and (b) the relation between antibody concentrations determined with different antigen preparations. Differences between subgroups of data were analyzed by pooled t-test or paired t-test, as appropriate.

Results and Discussion

Characteristics of Modified LDL

Typical TBARS concentrations were 0.28 nmol/100 µg of protein (2.8 µmol/g) for native LDL and 12.5 nmol/100 µg of protein (125 µmol/g) for mLDL, indicating that the mLDL preparations were fully oxidized (17). The preparations were apparently stable, giving consistent values for mLDL/LDL for at least 8 weeks as determined for three sera stored frozen at −20°C (slope = −0.0154, P = 0.41) (Fig. 1). Also, the TBARS concentration of the mLDL and LDL preparations did not change over 3 months of storage at 4°C (data not shown). Preparations of LDL and mLDL from the same volunteer were used in these experiments. There was variation in absolute values for serum concentrations of antibody against mLDL (expressed as mLDL/LDL) between two preparations (r = 0.6587, n = 18 sera); Salonen et al. (4) also reported variations. However, the preparations responded in the same way to changes in serum dilution (1:10–1:1000) (data not shown), indicating that reoptimization of assay conditions is not necessary for each new preparation of LDL and mLDL.

Assay Specificity

To confirm that serum IgG was binding specifically to mLDL, we determined whether free mLDL or LDL could compete for serum IgG binding to mLDL or LDL coated to the wells of the microtiter plates. Serum IgG binding to mLDL was measured by our ELISA (with HSA blocking buffer), except that 0–50 mg of LDL or mLDL protein was included in the incubation step. Consistent with the results obtained with a polyclonal antibody preparation (8), mLDL competed more effectively for serum IgG binding to coated mLDL than LDL (Fig. 2). Neither mLDL nor LDL (0–50 mg) reduced serum IgG binding to coated LDL. The data in Fig. 2 represent the findings for a single serum; similar findings (not shown) were obtained with a different serum when we used BSA for blocking.

Different Blocking Agents

BSA (d) and HSA (8, 9) have been used as blocking agents in previous studies of IgG binding to mLDL. Because 6.5% of sera contain significant IgG reactivity against BSA and other commonly used blocking agents (18), the characteristics of the blocking buffer could confound estimates of IgG binding to mLDL. We therefore investigated three different blocking agents: BSA (10 g/L in PBS), HSA (5 g/L in PBS), and Superblock. In the present system, the HSA concentrations reported by Vaarala et al. (9) (20 g/L HSA in PBS for blocking and 2 g/L HSA in PBS for serum and enzyme-conjugated antibody diluent) obliterated any absorbance signal. However, adequate signal was recovered when HSA was omitted from the serum and conjugate diluent. The concentration of 5 g/L HSA in PBS was chosen arbitrarily, there being no apparent differences from 1 to 20 g/L HSA (data not shown).

Comparative data for the three blocking buffers are presented in Table 2; 18 sera randomly selected from samples entering our laboratory for clinical testing were assayed. These experiments were performed with optimal experimental conditions for each blocking buffer, as given in Table 1. Wash buffer contained 1 g/L BSA when BSA was used for blocking because omitting BSA from the wash buffer caused substantial decreases in mLDL/LDL binding ratio.
LDL with increasing serum dilution (data not shown). mLDL/LDL was significantly higher with Superblock than with HSA or BSA (P < 0.001). The lowest amount of binding to LDL, which is the best available estimate of nonspecific binding, was obtained with HSA; the highest binding was with BSA. Interestingly, the relation between different approaches to quantifying serum antibody concentrations differed between blocking buffers. As illustrated in Fig. 3, when Superblock was used for blocking, the correlation between mLDL/LDL and normalized mLDL−LDL values was relatively poor (r = 0.494, P = 0.037), but correlations were excellent with BSA (r = 0.978, P < 0.001) or HSA (r = 0.878, P < 0.001).

Taken together, these data suggest that HSA is the blocking buffer of choice, given that it has the lowest nonspecific binding (as estimated by IgG binding to LDL), together with a significant correlation between data obtained by using different methods of calculating antibody concentrations, as would be expected if nonspecific binding was minimized. Therefore, detailed optimization data are presented below for HSA alone, except where data for BSA or Superblock are of particular relevance. Expression of our data as mLDL−LDL rather than as mLDL/LDL did not affect the conclusions of the optimization experiments (data not shown). We took into account both mLDL/LDL (as a measure of signal/noise) and the high value/low value (as a measure of the relative ranges of values attainable).

### Table 2. Effect of blocking buffer on serum IgG binding to mLDL and to LDL

<table>
<thead>
<tr>
<th>Blocking buffer</th>
<th>mLDL/LDL</th>
<th>LDL binding (A₄₅₀)</th>
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<tbody>
<tr>
<td>5 g/L HSA in PBS</td>
<td>1.72 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.087 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 g/L BSA in PBS</td>
<td>1.26 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.877 ± 0.142&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Superblock</td>
<td>3.95 ± 0.87</td>
<td>0.169 ± 0.048</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from BSA and Superblock; P < 0.001 by paired t-test.
<sup>b</sup> Significantly different from HSA and Superblock; P < 0.001 by paired t-test.

Optimization Studies

**Antigen concentration.** The effect of antigen concentration was investigated by comparing the IgG binding to LDL, mLDL, and the antigen blank over a range of antigen concentrations; serum and enzyme-conjugated second antibody were diluted 1:250 and 1:5000, respectively. Both mLDL/LDL and high value/low value increased with increasing antigen concentration, up to 25 mg/L (Fig. 4A).

During the course of these experiments, we observed that, as the antigen concentration increased, the amount of antibody binding to LDL decreased to less than the binding to the antigen blank. At antigen concentrations of 1 and 50 mg/L, respectively, the IgG binding to LDL/IgG binding to the antigen blank were 1.51 ± 0.17 and 0.46 ± 0.09 (mean ± SD) for HSA (n = 7, P < 0.001). Similar data were obtained for Superblock (1.03 ± 0.08 and 0.78 ± 0.22, n = 8, P < 0.05) and for BSA (1.01 and 0.83, n = 1). These data suggest that the lipoproteins are to a certain extent self-blocking, and that IgG binding to the antigen blank overestimates the amount of nonspecific binding in the wells containing LDL or mLDL. Clearly, comparisons of IgG binding to LDL or mLDL with binding to the antigen blank is not appropriate for quantifying the IgG reactivity.

Reductions in nonspecific binding would explain the somewhat anomalous increase in mLDL/LDL with antigen concentration. In theory, the ratio should not change, because the increases in LDL and mLDL concentration were the same; however, progressive decreases in nonspecific binding result in increases in mLDL/LDL with increasing antigen concentration. Thus, the data also indicate that differences in the absolute values of IgG binding to LDL or mLDL (as A₄₅₀) at different antigen concentrations cannot be used as a direct measure of changes in the amount of specific binding.

The optimal antigen concentrations identified here are considerably higher than those used by others; most previous assays have used an antigen concentration of 5

![Fig. 3. Effect of blocking buffer on relation between the two methods of data expression. IgG binding to mLDL was measured in 18 sera with 5 g/L HSA in PBS (left), 10 g/L BSA in PBS (middle), or Superblock (right) as blocking buffer. Results of linear regression analysis are shown for each comparison.](image-url)
mg/L (4, 9, 13), although Parums et al. (6) used 40 mg/L. As discussed above, nonspecific binding is reduced at higher antigen concentrations.

**Serum dilution.** To determine the optimal serum dilution for the assay of serum antibodies against mLDL, we examined mLDL/LDL over a range of serum dilutions; antigen concentration was 25 mg/L and the enzyme-conjugated second antibody was diluted 1:5000. mLDL/LDL increased with increasing serum dilution, to a maximum at 1:100 (n = 6); however, the ratio between the high value and the low value was greatest at 1:250 (Fig. 4B). Therefore, 1:250 was chosen as the serum dilution for further experiments with HSA as blocking agent. With BSA, there was no increase in mLDL/LDL with increasing serum dilution (n = 8, data not shown); therefore, the dilution of 1:100 was chosen arbitrarily for further experiments.

**Dilution of enzyme-conjugated second antibody.** mLDL/LDL was measured over a range of dilutions of enzyme-conjugated second antibody (HRP-conjugated rabbit anti-human IgG Fc); the antigen concentration was 25 mg/L and the serum dilution was 1:100. The concentration of undiluted enzyme-conjugated second antibody was 1.0 g/L. Maximum values for mLDL/LDL were reached at a dilution of 1:5000 (n = 7); there was no apparent trend in the high value/low value ratio with enzyme-conjugated second antibody dilution (Fig. 4C).

**Precision**

To the best of our knowledge, the question of assay precision (CV) has not been addressed in previous reports. We therefore examined both within-run and between-run precision (HSA blocking buffer, optimized experimental conditions) with six serum samples with a range of antibody concentrations. All sera were stored in aliquots at -20°C before assay. As illustrated in Table 3, the best precision was obtained when the data were expressed as mLDL/LDL rather than as mLDL-LDL. Normalization of the absorbance data significantly improved between-run precision when data were expressed as mLDL-LDL, although the value remained high (CV 30.5%) compared with between-run precision for mLDL/LDL (CV 8.9%).

**IgG Antibodies Against mLDL in SLE Patients and Controls**

To examine the effect of different assay methodology and methods of data expression on study outcome, we
assayed IgG antibodies against mLDL in sera from 20 subjects with SLE and 20 controls, using either 5 g/L HSA, 10 g/L BSA, or Superblock as blocking agents. Measurable concentrations of these antibodies were found in all sera tested, consistent with the findings of others (7, 8).

Increased concentrations of anti-cardiolipin IgG antibodies were observed in five of the SLE subjects and in two controls. Four of the SLE subjects with anti-cardiolipin antibodies also had increased amounts of anti-phosphatidyl serine IgG. Although the numbers are too few for statistical analysis, there was no trend to indicate a difference in concentrations of anti-oxidized LDL antibodies between subjects with and without anti-cardiolipin antibodies. For subjects with SLE (with HSA for blocking), the mean (± SD) mLDL/LDL was 1.64 ± 0.20 (n = 5) for those with anti-cardiolipin antibodies and 1.64 ± 0.23 (n = 15) for those without; corresponding data in the control group were 1.42 (n = 2) and 1.37 ± 0.14 (n = 18). This contrasts with other findings reporting an association between amounts of anti-cardiolipin and anti-oxidized LDL antibodies (9).

Vaarala et al. (9) reported that concentrations of antibody to oxidized LDL were higher in SLE patients than in controls (data expressed as mLDL/LDL: HSA for blocking). Our results (Table 4) agreed with this, depending on the means of data expression and (or) blocking buffer used. When HSA was used for blocking, antibody concentrations were higher in SLE patients than in controls for both methods of data expression (P <0.01). However, when BSA or Superblock were used, antibody concentrations were significantly higher in SLE subjects only when data were expressed as mLDL/LDL. This finding is most probably an effect of nonspecific binding on the mLDL/LDL calculation: High nonspecific binding causes a decrease in mLDL/LDL and, as we have demonstrated, Superblock and 10 g/L BSA are less effective than 5 g/L HSA in reducing nonspecific binding. Thus, in a study where there are differences in nonspecific binding between patients and controls (as is very likely with SLE), differences in the amounts of IgG antibody against mLDL between groups may not be adequately assessed when the data are expressed as mLDL/LDL, unless HSA is used for blocking. The methods of Vaarala et al. (9) and of Virella et al. (7) are not affected by differences in nonspecific binding.

Our approach, which tries to minimize nonspecific binding and expresses antibody concentrations as mLDL/LDL, has certain potential limitations. The underlying assumption of this calculation is that the two LDL preparations, native and malondialdehyde-modified, give the same degree of nonspecific binding. If this assumption is false, there would be a systematic bias between the estimate of autoantibody concentration by mLDL/LDL and the true concentration. Furthermore, mLDL/LDL can be confounded by differences in nonspecific binding between samples; the use of HSA for blocking minimizes this problem.

In summary, in this method for assaying concentrations of serum antibody against oxidized LDL, the precision is best when the data are expressed as mLDL/LDL; however, this calculation is potentially affected by variations in nonspecific binding among sera. Because the choice of blocking buffer and the method of data expression might affect experimental findings, we suggest a cooperative effort to formally compare and (or) standardize methodology. Further work is needed to characterize the exact nature of these autoantibodies against mLDL and the epitopes (in vitro and in vivo) with which they react.

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References