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Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers

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Summary. We have developed a solid-phase enzyme immunoassay for anti-oxidized low-density lipoprotein antibodies. Most sera showed some degree of non-specific binding to plates coated with oxidized low-density lipoprotein and the autoantibodies to oxidized low-density lipoprotein often appeared to have a relatively low affinity. To differentiate between specific and non-specific binding each sample was tested untreated and after absorption with oxidized low-density lipoprotein. The optical densities obtained with dilutions of the absorbed sample were considered to reflect non-specific binding and were subtracted from values obtained with identical dilutions of the unabsorbed sample, to yield corrected values from which the concentrations of anti-oxidized low-density lipoprotein antibody were calculated. Similar absorptions with native low-density lipoprotein and oxidized human serum albumin failed to induce a significant reduction in binding to immobilized oxidized low-density lipoprotein proving that the antibodies measured by this assay are primarily specific for oxidized low-density lipoprotein. We studied sera from two groups of individuals: (1) 33 subjects submitted to coronary angiography and split into two subgroups depending on the degree of coronary stenosis and (2) 64 healthy individuals also split into two subgroups according to lipid levels. Anti-oxidized low-density lipoprotein antibodies were detected both in patients and healthy individuals. Higher levels were detected in patients with moderate coronary disease and hyperlipemic healthy individuals, but the differences between patients and healthy volunteers or between their respective subgroups did not reach statistical significance. Our results suggest that autoantibodies to oxidized low-density lipoprotein are relatively frequent in both symptomatic and asymptomatic individuals. The investigation of their potential role as a risk factor will require mass screening and long-term follow-up.

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Introduction

The role of oxidized low-density lipoprotein (ox-LDL) in the pathogenesis of atheroslerosis has been the object of intense investigation. Ox-LDL has been found to stimulate macrophage transformation into foam cells, to induce endothelial cell (EC) functional changes, and to be cytotoxic [17, 30]. Additionally, ox-LDL can trigger can autoimmune response leading to the formation of antibodies [26]. Antibodies to LDL and modified LDL and immune complexes apparently involving LDL as the antigenic moiety (LDL-IC) have been demonstrated in patients with coronary disease, diabetics, and normal subjects by a variety of techniques [10, 16, 18, 20, 22, 23, 25, 28, 29]. The pathogenic role of LDL-IC has been suggested by experiments demonstrating that incubation of cells with LDL-IC significantly disturbs lipoprotein and cholesterol metabolism. The first observation was made by Dachet et al. [1] who showed that the exposure of human fibroblasts to LDL-IC resulted in excess production of free cholesterol. Later Klimov et al. [14] showed excessive cholesteryl ester (CE) accumulation in mouse peritoneal macrophages exposed to LDL-IC, and recently Orekhov et al. [18] showed increased intracellular cholesterol accumulation in cultures of human subendothelial cells exposed to LDL-IC prepared with native and modified LDL and anti-LDL antibodies purified from sera found to enhance foam cell formation. We have extensively studied this phenomenon using human monocyte-derived macrophages and have concluded that incubation with LDL-IC is more efficient that any other known protocol for the induction of foam cell formation *in vitro* [11, 12]. In addition, the incubation of monocyte-derived macrophages with LDL-IC triggers the release of cytokines, such as tumor necrosis factor-α and interleuk-

in-1 (G. Virella, M. F. Lopes-Virella, unpublished observations), which have the potential of contributing to smooth muscle cell proliferation and EC damage.

The hypothesis that LDL-IC play a pathogenic role in atherosclerosis is supported by the evidence collected by several groups showing that anti-LDL antibodies may be formed *in vivo*. For example, it has been shown that oxidation, methylation, ethylation, acetylation, carbamylation, and non-enzymatic glycation apoliprotein-B (Apo-B) induce the formation of antibodies to the modified apoprotein [27, 31]. Glycation, oxidation, and malondialdehyde (MDA) modification of lipoproteins have been shown to occur *in vivo* [19, 24, 27] and antibodies directed against glycated LDL have been indirectly demonstrated in diabetic patients [31].

Circulating anti-LDL antibodies in patients with coronary artery disease have been described by several independent groups [3, 10, 18, 28, 29]. Thus far, the methods used for detecting free circulating antibodies to LDL have induced polyethylene glycol (PEG) precipitation, ultracentrifugation followed by detection of immunoglobulins in the LDL layer, solid-phase radioimmunoassays, passive hemagglutination, and affinity chromatography [10, 16, 18–20, 22, 23, 26, 28, 29]. These methods suffer from several drawbacks, such as lack of specificity and precision in the case of PEG precipitation and detection of IgG in the LDL separated by ultracentrifugation, poor reproducibility in the case of passive hemagglutination, and the problems of short shelf-life and disposal of contaminated materials inherent to radioimmunoassays. Also, several of these approaches do not allow the investigation of whether the anti-LDL antibodies react with native or with modified LDL. Therefore, we have developed an enzyme immunoassay (EIA) for the detection of free antibodies to modified lipoproteins which appears to have advantage over all previously published techniques.

Patients and methods

Patients and samples. We studied a total of 97 subjects, divided into two major groups: (1) 64 asymptomatic normal healthy volunteers and 33 patients with angiographically confirmed obstructive coronary heart disease (CHD). Coronary arteriography was performed using the Judkins (percutaneous femoral) or Sones (brachial arteriotomy) approach. Multiple views of the left and right coronary arteries were obtained and a permanent recording made using cineangiography. The arteriograms were analyzed by two cardiologists. The group of patients with CHD was subdivided into two sub-groups: patients with a degree of coronary stenosis as seen in coronary angiography of 50%–70% in at least one vessel were classified as having moderate CHD; those with stenosis greater than 70% in at least one vessel were classified as having severe CHD. The group of healthy volunteers was also subdivided into two subgroups depending on whether the levels of serum lipids and lipoproteins were within normal limits or not (levels of LDL cholesterol above 160 mg/dl and levels of triglycerides above 250 mg/dl were considered abnormal).

Blood was collected by venipuncture, clotted at 37 °C for 1 h, and immediately centrifuged to separate serum, to which sodium EDTA (1 mg/ml) was immediately added. The serum was stored in the refrigerator prior to assay. Antibody assays were carried out not later than 10 days after blood collection.

Lipoprotein isolation and modification. Blood for lipoprotein isolation was collected in EDTA (1 mg/ml) after 12 h of fasting. LDL ($1.019 < \text{density} < 1.063$) was isolated from plasma, after density adjustment with potassium bromide, by preparative ultracentrifugation at 50,000 rpm/min for 22 h on a Beckman L5-50 ultracentrifuge, using a type 50 rotor [15]. LDL preparations were washed by ultracentrifugation, dialyzed against a 0.15 M sodium chloride solution containing 1mM EDTA, pH 7.4, passed through an Acrodisc filter (0.2 μm pore size) in order to remove aggregates, and stored under nitrogen in the dark. Oxidation of LDL was performed using the protocol described by Steinbrecher et al. [27] which consists of incubating isolated LDL diluted in Ham's F-10 medium with 10 mM copper (Cu^{2+}) at 37 °C for 24 h. The process was stopped by the addition of 200 mM EDTA and 40 mM butylhydroxytoluene (BHT), and the ox-LDL was dialyzed against phosphate-buffered saline (PBS) containing 200 mM EDTA and 40 mM BHT. The degree of oxidation of the LDL preparation was measured by the TBARS assay [13] and found to be quite consistent in different batches (7.521 ± 1.1638 nmol malondialdehyde (MDA) equivalents/mg protein). We considered ox-LDL preparations with TBARS values ranging between 6 and 9 nmol MDA equivalents/mg cholesterol acceptable for the assay. This degree of oxidation is associated with physical and biological properties analogous to those of LDL-like particles isolated from atheromatous lesions, as described by Ylä-Herttuala et al. [32].

EIA of anti-LDL antibodies. Flat-blottomed Immulon type 1 plates were coated with ox-LDL in 0.25 M sodium carbonate buffer pH 9.6 (100 μl per well of a 5 μg/ml ox-LDL preparation) and incubated overnight at 4 °C. The unbound ox-LDL was then washed off, and the plates were blocked with 5% bovine serum albumin (BSA) in PBS pH 7.4 (200 μl-well, 45 to 60-min incubation at 37 °C). After blocking, the plates were washed three times in PBS with 0.05% Tween 20 (Sigma).

Serum samples were run both adsorbed with ox-LDL (100 μg/ml) and unabsorbed. The samples were prepared the day before the EIA was run, as illustrated in Fig. 1. The absorbed samples were first diluted 1:5 in PBS with 1% BSA (PBS-BSA) and then 1:2 with native or ox-LDL. The unabsorbed samples were diluted 1:10 in PBS-BSA. All samples (absorbed and unabsorbed) were incubated on a blood rocker at 4 °C overnight and spun at 5,000 rpm for 10 min the next morning to discard any precipitable aggregates and antigen-antibody complexes. Very little or no precipitation was seen after centrifugation. The supernatants of the centrifuged samples were carefully pipetted, avoiding drawing the bottom 50–100 μl, and diluted serially with PBS-BSA. Aliquots of the dilutions (100 μl) were transferred to the blocked and washed microtiter plates. The plates were then incubated for 2 h at 37 °C and overnight at 4 °C. The next morning, the plates were washed four times with PBS-Tween 20, followed by the addition of 100 μl/well of a 1:4,000 dilution of peroxidase-labeled rabbit anti-human whole IgG (Cappel) in PBS-BSA. The conjugate was incubated for 1 h at room temperature. The plates were washed three times with PBS-Tween 20 and 100 μl of substrate 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) in citric acid buffer to which we added 3% (v/v) hydrogen peroxide [immediately before use] was added to each well. The reaction was allowed to proceed until the intensity of the color reaction was satisfactory, which usually took 10 min. The reaction was stopped with 100 μl/well of 0.1 M citric acid and the color intensity was read on a Molecular Devices EIA reader set at 414 nm.

The assay was calibrated with serial dilutions of IgG isolated from a rabbit hyperimmune anti-LDL antiserum prepared in our laboratory, starting at 200 ng/ml. With this control we used peroxidase-labeled sheep anti-rabbit IgG antibody at a 1:8,000 dilution. The antibody concentration for the unknowns was determined by calculating the mean of the values extrapolated from the calibration curve using the difference between the optical density (OD) with untreated and ox-LDL absorbed aliquots of 1:10 and 1:20 dilutions. The best fit for the calibration curve was obtained with a second degree polynomial function. The slopes of the calibration curve constructed with rabbit anti-LDL IgG and of the OD versus dilu-

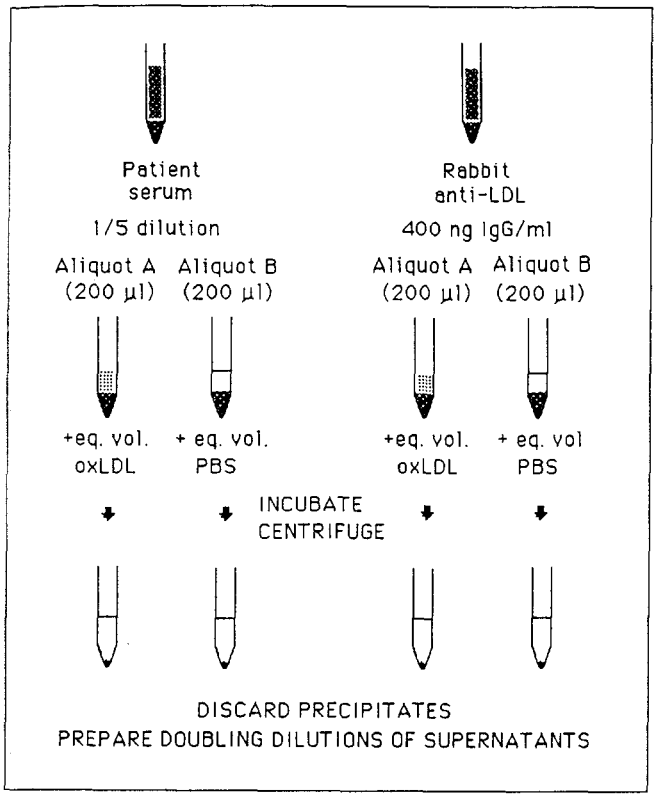

Fig. 1. Diagrammatic representation of the protocol used to adsorb serum samples with oxidized low-density lipoprotein (*ox-LDL*). *PBS*, phosphate-buffered saline; *eq. vol.*, equal volume

tion plots obtained from the data generated with patient sera were not identical; therefore the values calculated for the human samples can only be considered as indicators of antibody concentrations. This allows comparisons between different samples but cannot be considered as accurately reflecting the antibody mass in each sample. Thus, we adopted the designation of nanogram equivalents per milliliter for the concentrations of human antibody, endeavoring to make clear that the figures represent OD equivalents to known concentrations of rabbit IgG with anti-LDL antibody activity.

Results

Conditions and performance of the EIA for anti-LDL antibodies

Several variables were considered before we established optimal conditions for the assay of autoantibodies to modified lipoproteins, particularly the type of modified LDL to use, the isotype of antibody to detect, and the possibility that the apparent concentration of anti-LDL antibodies could be affected by the spontaneous association of the antibodies with ox-LDL spontaneously generated during storage.

Several types of in vitro-modified LDL preparations have been studied with respect to their potential involvement in the pathogenesis of atherosclerosis. Recent studied have emphasized the potential pathogenic role of ox-LDL; LDL with variable degrees of oxidation has been recovered from atheromatous lesions [19, 33] and detected in peripheral blood [6, 24]. Ylä-Herttuala et al. [32]

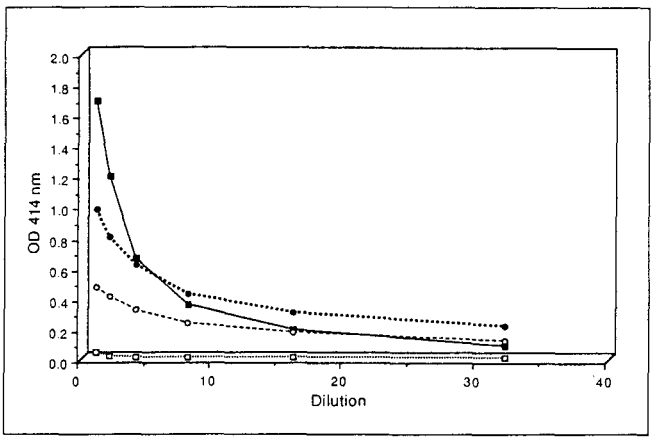

Fig. 2. Diagrammatic representation of the reduction in reactivity with immobilized ox-LDL seen after adsorption of rabbit anti-LDL and of a positive human serum with ox-LDL. —■—, Unabsorbed rabbit anti-LDL; ····□····, absorbed rabbit anti-LDL; ---●---, unabsorbed human serum; ---○---, absorbed human serum; *OD*, optical density

compared the physical and biological characteristics of copper sulfate-oxidized LDL and LDL-like particles isolated from the atherosclerotic intima, and found them to be remarkably similar. Hence we decided to use copper sulfate oxidized LDL as the antigen in our assay for anti-LDL antibodies.

The question of which is the most significant antibody isotype in autoimmune responses to LDL has not completely been resolved, since there is anecdotal evidence favoring the involvement of both IgA and IgG [2, 7, 8, 21, 23]. Thus, we decided to use a polyvalent anti-IgG antibody, which will react with both IgG and IgA antibodies, and hence establish an assay for total antibody. Finally, we ran a series of assays varying the concentrations of ox-LDL used for coating the microtiter plates and for absorption of anti-ox-LDL in the control and unknown samples, as well as the concentrations of fluorescein-labeled anti-immunoglobulin. The concentrations of antigen and labeled antibodies were established in preliminary studies using different concentrations of reactants. The dilutions of rabbit antibodies and of a human positive control serum (kindly provided by Dr. Walter Riesen, Bern, Switzerland, and known to contain anti-LDL antibodies capable of agglutinating LDL-coated red blood cells to a titer of 512 and of precipitating LDL in a double diffusion assay [23]) giving optimal dose-response curves were determined.

The specificity of the assay for heterologous anti-human LDL antibodies is clearly demonstrated by the observation that the reactivity of IgG isolated from a hyperimmune anti-LDL rabbit serum was virtually abolished by absorption with ox-LDL (Fig. 2). The concentration ox-LDL chosen for sample absorption (100 µg/ml) was fourfold in excess of the minimal concentration capable of absorbing 90% of the binding activity of our reference rabbit anti-LDL antibody containing 200 ng/ml of IgG. Identical reductions in the reactivity of rabbit anti-LDL were observed when the antiserum was pre-absorbed with ox-LDL and native LDL, demonstrating that the

Table 1. Results of a cross-absorption study with oxidized low-density lipoprotein (ox-LDL), native LDL, and oxidized human serum albumin (ox-HSA)[a]

Subject	OD reduction after absorption with ox-LDL	OD reduction after absorption with native LDL	OD reduction after absorption with ox-HSA
1	17.5±2.5%	3±1%	4±2%
2	11±3.5%	2.5±2.5%	1.5±5%
3	11±1%	1±0%	1±1%
4	11±5%	4±1%	4±5%
5	12±4%	0±3%	−2±5%
6	11±6%	7±4%	1±1%
7	13±1%	7±1%	9±1%
8	15±1%	8±2%	9±3%
9	22±0%	10±1%	8±1%
10	16±3%	1±2%	4±2%
Mean	14±4.5%	4±4.2%	3.8±4.5%

[a] Mean±1 SD based on three determinations at three different dilutions

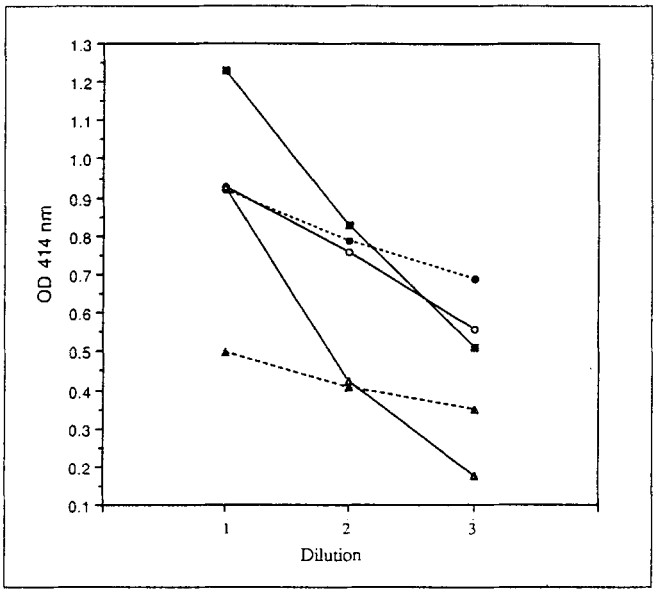

Fig. 3. Comparison of the slopes obtained plotting OD versus dilution of samples of rabbit anti-LDL (—⊞—) and of two different human anti-ox-LDL antibody-containing sera with high levels of anti-LDL antibody (697 ngEq/ml for patient A and 909 ngEq/ml for patient B). For each human serum two plots are shown: one corresponds to the difference in OD between the native sample and the sample absorbed with ox-LDL (—○—, patient A; —△—, patient B), which corresponds to the "true" antibody fraction; the other corresponds to the residual binding of the absorbed sample (---●---, patient A; ---▲---, patient B), which should reflect non-specific binding

rabbit hyperimmune serum contains antibodies that react with apoprotein determinants not affected by oxidation. In the case of human sera found to have high binding activity to ox-LDL, absorption with ox-LDL caused a significant, but incomplete, reduction in binding (Fig. 2). Pre-incubation of serial dilutions of the human positive control with ox-LDL resulted in a 50% decrease in reactivity. The greatest reduction seen in a human sample was 75% in a serum estimated to contain 400 ng/ml of anti-ox-LDL antibody.

Since heterologous anti-LDL antibodies reacted equally well with native and ox-LDL, it was important to study in further detail the specificity of the human autoantibodies detected with this assay. Hence we compared the effects of pre-incubating a randomly chosen group of 10 sera found to have significant binding activity to immobilized ox-LDL with ox-LDL, native LDL, and oxidized human serum albumin (ox-HSA). This represented about 40% of the samples that had antibody levels in excess of 200 ngEq/ml. The ox-LDL used in this study was obtained from the native LDL used as a control, and the ox-HSA was prepared at the same time and under identical conditions as the ox-LDL. All proteins were used at identical concentrations in the absorption step (100 μg/ml). As shown in Table 1, pre-incubation with ox-LDL was most effective in reducing reactivity with immobilized ox-LDL. The mean OD reduction caused by adsorption of these 10 samples with ox-LDL was 14±4.5%, while absorption with native LDL and ox-HSA caused a reduction of only 4±4% and 3.8±4.5%, respectively. Statistical analysis by the paired *t*-test showed a significant difference between the OD reduction caused by absorption with ox-LDL and the reduction caused by absorption with any of the other two proteins ($P=0.0001$), while there was no significant difference between absorption with native LDL and ox-HSA ($P=0.637$). Analysis of the results obtained with individual samples suggested that 2 may have contained cross-reactive antibodies absorbable with native LDL (subjects 8 and 9) and 3 may have contained antibodies cross-reactive with oxidized radicals in HSA (subjects 7, 8 and 9), but in all samples the predominant reactivity appeared to be with unique determinants of ox-LDL.

The relatively high level of residual reactivity seen in many positive sera after absorption with ox-LDL could reflect either non-specific binding of IgG to ox-LDL or the predominance of low affinity antibodies to ox-LDL. The comparison of plots of OD for the binding of samples absorbed with ox-LDL (believed to correspond to non-specific binding) with plots of the values for the difference in OD between unabsorbed and absorbed samples of the same serum (believed to reflect the "true" antibody fraction), using the plot of OD values obtained with dilutions of rabbit anti-LDL antiserum (containing high-affinity anti-LDL antibodies) as reference (Fig. 3), strongly suggests that the human antibodies are of variable affinity, some of an affinity perhaps as high as that of the rabbit antibody, and some of considerably lower affinity. But in all cases, the slopes of the curves plotted with the OD values representing the differences in binding between absorbed and unabsorbed values were steeper than the slopes obtained plotting OD values for absorbed samples, supporting our contention that the absorption step removes specific antibody activity from the sample.

The differences in apparent binding affinity between rabbit antibody and human autoantibodies constitute a significant cause of error in the extrapolation of concentrations based on the reactivity of the control rabbit anti-

serum. For this reason we decided to base the calculation of anti-ox-LDL antibody concentrations on the mean values obtained from absorbed and unabsorbed aliquots of two dilutions (1:10 and 1:20) of each sample. In addition, the calibration of the assay was carried out with purified IgG from a rabbit hyperimmune anti-LDL antiserum of which only an undetermined proportion was specific antibody and two different conjugates had to be used for the calibration curve and the unknowns. Thus, the concentrations are given in nanogram equivalents per milliliter, i.e., the OD of human samples have been correlated with the OD obtained with purified IgG of a rabbit antiserum, and cannot be considered as an accurate determination of the absolute concentration of antibody. However, the calculated concentrations do reflect quantitative differences between samples obtained from different individuals.

The possibility that the assay could be affected by the auto-oxidation of LDL during storage was considered. For this reason, 1 mg/ml of sodium EDTA was added to all serum samples (which also contain naturally occurring anti-oxidant substances); the samples were stored at 4 °C immediately, after separation and addition of EDTA, and the assay of anti-LDL antibodies was carried out within 10 days of collection. These precautions should be sufficient to avoid oxidation of the lipoproteins contained in the sera, since auto-oxidation of isolated LDL in the presence of EDTA is an extremely slow process, which requires several months before it can be detected [5]. To further confirm that storage for up to 10 days was not interfering with the results, we repeated assays of 20 samples during the 10 days after collection. The mean concentration of anti-LDL antibody in these 20 samples was 210 ngEq/ml, and while in 8 samples there was a slight decrease in the values obtained in the second assay, in 12 other samples the second assay yielded higher values and the average variation was of +15 ngEq/ml (the opposite of what was expected if binding with auto-oxidized LDL was a significant confounding factor).

Anti-LDL antibody assay in controls and subjects with occlusive coronary disease

The assay of anti-ox-LDL antibodies in normal healthy volunteers and patients with occlusive (CHD) showed that there are wide ranges of variation in both groups, as summarized in Table 2. The highest mean values and the higher percentage of sera containing 200 ngEq/ml of anti-ox-LDL antibody or more was determined in hyperlipemic individuals, identified among our group of normal healthy volunteers. However, the range of anti-ox-LDL antibody concentrations in normal individuals was very wide and the differences between normolipemic and hyperlipemic healthy volunteers did not reach statistical significance. Among the groups of patients with angiographically documented coronariopathy, those with less severe lesions appeared to have higher levels of anti-ox-LDL antibody, but the number of patients in this group was relatively small and statistical analysis failed to show a significant difference between the two patient groups. Although the mean concentration of anti-ox-LDL antibody and the frequency of samples with concentrations of antibody of 200 ngEq/ml or more were considerably lower in patients with severe coronary stenosis than in hyperlipemic healthy volunteers, statistical analysis failed to reveal any significant differences when patients and controls were compared, either as single groups or as subgroups.

Table 2. Summary of the results obtained in 64 normal healthy volunteers and 33 patients submitted to coronary angiography[a]

	n	Mean	SD	Range	% ≥200
Healthy volunteers (total)	64	156	141	0–583	28
Healthy volunteers (normal lipids)	50	147	138	0–515	24
Healthy volunteers (hyperlipemic)	14	186	149	0–583	43
Patients (all)	33	143	201	0–1106	25
Patients (severe coronariopathy)	26	107	118	0–463	23
Patients (moderate coronariopathy)	7	269	384	32–1106	28

[a] Values expressed as nanogram equivalents per milliliter

Discussion

The possible pathogenic significance of anti-LDL antibodies was first suggested by Beaumont [2, 3], who reported a small group of patients with IgA multiple myeloma, whose paraproteins reacted with autologous LDL. The patients presented with hyperlipidemia and xanthomatosis, and the term *autoimmune hyperlipidemia* was coined to designate the clinical picture. Further investigations showed that atherosclerosis is the chief complication of autoimmune hyperlipidemia and that immunosuppressive treatment reduced both hyperlipidemia and accelerated atherosclerosis. Dachet et al. [7] further demonstrated that LDL complexed with monoclonal IgA anti-LDL would bind normally to fibroblasts but its internalization was defective. These observations were given additional dimension by the description by others of autoantibodies to LDL in patients with coronary artery disease of all ages [10, 18, 20, 29]. While the work of Beaumont deserves to be considered as seminal in this field, it needs to be recognized that it was based on a few very exceptional cases of monoclonal proteins with anti-LDL activity and it also must be stressed that neither Beaumont nor any of the other pioneers in this area made any effort to characterize the epitopes of LDL recognized by anti-LDL autoantibodies. Thus, the impressions that IgA is often involved in LDL autoimmunity and that reactivity is directed to unmodified LDL are probably erroneous and need to be re-evaluated with more refined techniques.

The formation of antibodies to autologous LDL fulfills the classical criteria of an autoimmune response and it is likely to be triggered by structural modifications of the LDL molecule which seem to take place *in vivo*. Stein-

brecher et al. [26] demonstrated that several types of modifications of LDL render the molecule immunogenic in experimental animals. The antibodies were directed specifically against a modified lysine residue of apo-B. Therefore, a modification as small as the methylation of a lysine residue of homologous LDL can induce an autoimmune response. The same seems to be true in the case of oxidized or MDA-modified LDL [19, 24]. Also, Witzum et al. [31] documented the accelerated catabolism of glycated LDL in diabetics, which they attributed to the existence of circulating antibodies specifically reactive with this type of modified LDL.

As initially suggested by Beaumont [2, 3], one of the major consequences of the production of autoantibodies to modified LDL is profound alterations in the rate of clearance of LDL from plasma, as later corroborated by Witzum et al. [31]. However, it was soon noted that the clearance of LDL complexed with autoantibodies could also be increased, probably as a result of immune complex uptake by macrophages. This uptake depends on several factors, but mainly the antibody isotype and the size of the aggregates [21]. The nature of the antigenic determinants recognized by the antibodies to LDL also appears significant in determining the rate of clearance of the immune complexes. According to Riesen and Noseda [22] patients with anti-LDL antibodies reacting with the the apoprotein part of the molecule are hypolipemic, while patients with antibodies reacting with lipid determinants (probably phospholipids) are hyperlipemic. The reason for this different clearance rate has not been elucidated, but it must reflect impaired uptake of LDL-IC involving anti-phospholipid antibodies by both LDL and Fc receptors. The reaction with LDL receptors could be inhibited in all cases both by the modification of LDL [1] and by the association with anti-LDL antibody, but it is less clear what inhibits phagocytosis via Fc receptors when the antibodies react with phospholipids, since the nature of the recognized epitope should not affect the interaction of the Fc fragment with its receptors on monocyte-derived macrophages.

In our laboratory, we have documented the capacity of LDL-IC prepared *in vitro* with heterologous IgG anti-LDL antibodies reacting with apo-B determinants to induce CE accumulation in human macrophages. The uptake of these immune complexes prepared *in vitro* is predominantly mediated by phagocytosis through Fcγ receptors, but some LDL-IC are also taken up through LDL receptors. A major abnormality in LDL metabolism, induced by the uptake of LDL-IC, is the persistent upregulation of the LDL receptor, enabling the continuous uptake of LDL even in the presence of increased intracellular concentrations of free cholesterol and CE [11, 12]. Thus, LDL-IC seem to induce a unique state of metabolic disequilibrium in macrophages, characterized by increased accumulation of free cholesterol and CE, part transported by the ingested LDL and part resulting from *de novo* CE synthesis through acyl CoA: cholesterol transferase (ACAT), while the expression of LDL receptors remains paradoxically upregulated.

The significance of all the *in vitro* evidence which has been accumulated supporting the pathogenic potential of LDL-IC in human atherosclerosis hinges heavily on the demonstration of the occurrence of anti-LDL antibodies *in vivo*. Our findings agree, to some extent, with those reported by others who described anti-LDL antibodies in patients with hyperlipemia and/or atherosclerosis [2, 3, 10, 20, 22, 25, 28, 29] and are strongly supported by the data recently reported by Orekhov et al. [18]. These authors demonstrated that LDL-IC prepared with human antibodies reacting with apo-B induce intracellular cholesterol accumulation in cultures of human aortic subendothelial cells. However, a difference between our results and those reported by other groups is that we found no significant differences in the reactivity of sera from healthy volunteers and patients with confirmed coronary vascular disease. This was not an effect of age, since neither our patient population – or our asymptomatic volunteers were of advanced age. Some of our healthy volunteers who tested positive were in their late twenties or early thirties.

Our results suggest that the synthesis of anti-LDL autoantibodies is likely to be triggered by the oxidation of LDL, which appears to take place *in vivo*, probably in the arteriosclerotic plaque [19]. The fact that anti-ox-LDL antibodies are relatively frequent among asymptomatic, young individuals may indicate that they are involved in a physiological process whose purpose is to eliminate modified LDL particles, and the fact that concentrations of antibody to ox-LDL appear to be greater in hyperlipemic individuals suggests that hyperlipemia may be associated with the formation of larger quantities of ox-LDL. It could thus be speculated that anti-ox-LDL antibodies represent an additional risk factor, particularly when present in high concentrations. Obviously, this possibility cannot be proven or disproven except by long-term follow-up studies. In addition, the lower concentrations of anti-ox-LDL in patients with more advanced coronary lesions also need to be confirmed and explained. Such a reduction in circulating antibody concentration can be interpreted in two ways: (1) either the circulating antibodies are circulating as immune complexes, not detectable in assays for free antibody, (2) or the anti-ox-LDL are being trapped at the level of the active vascular lesions, where deposition of LDL and modified LDL is taking place. The first hypothesis was indirectly supported by data reported by other groups who have claimed to detect circulating immune complexes in patients with artherosclerosis [9, 28, 29], but we have not been able to find evidence of circulating immune complexes in our patients with coronary vascular disease using a solid-phase C1q binding assay (results not shown). The second hypothesis is supported by our knowledge of the modification of LDL *in vivo* [19, 24, 27] and by the detection of ox-LDL on atheroma lesions [19, 33]. Obviously, the formation of immune complexes *in situ* is likely to play the most important pathogenic role and may not be reflected in the levels of circulating immune complexes. Finally, it is possible that the immune response to ox-LDL may be heterogeneous, some antibodies recognizing more extensively modified LDL and others recognizing molecules with minimal degrees of modification. This possibility would explain the difference between

our results and those recently published by Salonen et al. [25], which suggest that antibodies to MDA-modified LDL may correlate with progression of carotid atherosclerosis. This suggests that antibodies to different types of modified LDL may have different biological properties, perfectly illustrating the degree of complexity in this field.

In conclusion, our data suggest that autoantibodies to copper sulfate-oxidized LDL are quite frequent in both symptomatic and asymptomatic individuals, and that their persistence in high levels in the circulation may be characteristic of the early stages of atherosclerosis, while in later stages the antibodies may be predominantly fixed in the lesions. Further investigations are needed to confirm our data and to further clarify the significance of LDL autoantibodies in atherosclerosis.

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