

## MINIREVIEWS

### Lipoprotein Autoantibodies: Measurement and Significance

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Two decades ago, well before the inflammatory nature of arteriosclerosis was generally accepted, several groups reported the finding of immunoglobulins and various complement components in atheromatous plaques of humans and rabbits (12, 42, 69). A decade later apolipoprotein B (ApoB) extracted from atheromatous lesions was found to react with monoclonal antibodies against malondialdehyde (MDA)- and 4-hydroxynonenal (HNE)-lysine, two common modifications of oxidized low-density lipoprotein (oxLDL) (38, 73), and immunoglobulin G (IgG) isolated from rabbit and human atherosclerotic lesions showed reactivity with MDA- and copper-modified LDL (74). These seminal observations represent arguably the best evidence available for the involvement of modified LDL antibodies and in situ formed antigen-antibody complexes in the induction and/or perpetuation of chronic vascular inflammation. It also gave a solid rationale for expanding study of the immunogenic properties of modified forms of LDL and to the development of methods for their assay in serum.

From the immunological point of view, oxLDL has been studied in most detail. LDL oxidation affects both the lipid and protein components of LDL. Reactive aldehyde products result from the oxidation of polyunsaturated fatty acids and include MDA and 4-HNE, capable of attaching covalently to the  $\epsilon$ -amino groups of lysine residues of ApoB (49, 50, 73). These modifications are present in copper-oxidized LDL, which was found to have structural and functional properties similar to those of LDL isolated from atherosclerotic plaques (73) and to react with monoclonal antibodies produced in guinea pigs against MDA and HNE-lysine (38, 73).

Detailed investigations have also been carried out with advanced glycosylation end product-modified LDL (AGE-LDL). Advanced glycosylation involves a chain of chemical reactions that starts with the nonenzymatic addition of reducing sugars to protein amino groups (Schiff base, Amadori adducts). If the half-life of a protein is sufficiently long, additional reactions take place leading to the formation of a heterogeneous family of sugar-amino acid adducts collectively known as “advanced glycosylation end products” (AGE) (43). LDL, like most plasma proteins, is susceptible to AGE modification (45). AGE-modified proteins are immunogenic (18), a property that

has been used to great advantage for their detection in serum (35) and localization in tissues (33, 35).

Several groups dedicated considerable effort to develop assays for antibodies reacting with copper-oxidized LDL and/or with MDA-modified LDL (2, 4, 8, 9, 16, 17, 22–24, 28, 36, 39, 44, 51, 55, 56, 60, 72). oxLDL antibodies have been detected in the sera of healthy persons and patients with vascular diseases and have been isolated and characterized (29, 63). Autoantibodies to AGE-modified serum albumin and AGE-modified IgG have also been demonstrated in human sera, both from diabetic patients and from nondiabetic subjects (27, 54, 57). The characteristics of isolated AGE-LDL antibodies have been recently reported (68), and results suggesting that these antibodies are able to combine with circulating AGE-modified antigens and form soluble immune complexes (IC) have also been published (54).

Considerable uncertainty exists about the clinical significance of modified LDL antibodies. The uncertainty results from two sets of observations: animal experiments that have been interpreted as suggesting a protective role for oxLDL antibodies (13) and conflicting data obtained in clinical and epidemiological studies that tried to correlate levels of oxLDL antibodies in serum with different end points of arteriosclerosis. These discrepancies are likely to result from multiple factors, including individual variations in the immune response, affecting concentration, isotype, and avidity of the autoantibodies, and inaccuracy of the assays, highly influenced by differences in antibody avidity and by the presence of soluble IC. Indeed, the assays used by different groups, as well as those made commercially available, are quite heterogeneous in design and standardization, making comparison of data obtained by different groups rather difficult.

#### CHARACTERISTICS OF HUMAN AUTOANTIBODIES TO MODIFIED LDL

Human autoantibodies to oxLDL and AGE-modified LDL have been isolated by affinity chromatography and characterized in regard to their isotype distribution and avidity (29, 63, 68) (Table 1). These data are remarkable for the consistent predominance of IgG antibodies of the proinflammatory IgG1 and IgG3 isotypes. The data obtained with antibodies purified by affinity chromatography do not coincide either with data obtained by enzymeimmunoassay (EIA) (71) or with the results of cloning experiments performed on experimental animals. However, it must be noted that data obtained by EIA are

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TABLE 1. Characteristics of affinity chromatography-purified antibodies to modified LDL

Antibody (n)	Immunoglobulin concn (mg/liter)							$K_d$ (mol/liter)
	IgG	IgM	IgA	IgG1	IgG2	IgG3	IgG4	
Human oxLDL antibody (30)	88 ± 30	20 ± 11	2.4 ± 1.3	62 ± 23	6.2 ± 2.5	18 ± 11	0.4 ± 0.2	$(1.02 \pm 1.1) \times 10^{-8}$
Human AGE-LDL antibody (7)	44 ± 25	14 ± 7	2.6 ± 1.8	26 ± 15	3.1 ± 4.2	14.5 ± 9.6	ND <sup>a</sup>	$(0.48 \pm 0.25) \times 10^{-8}$
IgG from rabbit and human ApoB antibody (3)								$(9.34) \times 10^{-11}$

<sup>a</sup> ND, not detected.

not truly quantitative, and any conclusions about the relative predominance of IgM versus IgG are questionable. It is equally important that the reported high "affinity" (71) of IgM oxLDL antibodies, which could be used as an argument in favor of their protective role by allowing the formation of stable, harmless LDL-IC, needs to be considered cautiously. Indeed, what is measured is the overall avidity of IgM antibodies, and the calculations are affected by its pentameric nature (65). The high values for molecular avidity cannot be confused with high affinity of the individual binding sites. This bonus effect of polyvalent IgM antibodies becomes inoperative when IgG antibodies of higher affinity reacting with multiple epitopes of the antigen compete with pentavalent but monospecific IgM molecules.

The experiments that resulted in the exclusive cloning of IgM oxLDL antibody-producing cells were carried out in ApoE-deficient mice (40), and extrapolating findings obtained with genetically modified mice to humans should be done with great reservations, particularly when the only reported attempt at cloning anti-LDL-producing clones from human subjects resulted in the isolation of an IgG-producing clone (47). Although protective properties were also attributed to this cloned IgG antibody, the clone in question did not secrete complete molecules but rather Fab fragments, totally devoid of opsonizing and complement-fixing abilities, lacking the biological properties of the intact antibodies (65).

The predominance of IgG2 antibodies to oxLDL reported by Wu and Lefvert (71) also needs to be interpreted cautiously because the data were obtained by EIA, and the assay of IgG2 by that technique is rather inaccurate (19, 20). In our laboratory we had the opportunity to compare the results of IgG2 assays in the same samples by radial immunodiffusion and EIA and verified that the latter values were two- to fivefold higher (Table 2).

The uniform predominance of IgG over IgM antibodies in the case of both oxLDL antibodies and AGE-LDL antibodies is important in the context of the current discussion about the

protective versus deleterious effects of modified lipoprotein antibodies (11, 15, 16, 41, 66, 67). However, the data generated by the characterization of affinity chromatography-purified oxLDL antibodies are clear in one respect: the predominant antibody isotypes are IgG of subclasses 1 and 3, and such antibodies are highly unlikely to play a protective role.

### ASSAY OF SERUM ANTIBODIES TO MODIFIED LDL

The vast majority of publications concerning the assay of antibodies to modified lipoproteins are based on direct binding EIA. However, there is a remarkable degree of heterogeneity in the EIAs developed by different groups and commercial sources (Table 3). Besides the basic differences between competitive and direct binding assays, there are significant differences in the type of oxidized LDL used by different groups, the control of modification degree, the standardization and calibration of the assays, and the calculation of the assay results.

Copper oxidation of LDL and, to a greater degree, MDA modification of LDL result in significant electric charge modifications of LDL. The increased negative charge is associated with an increased risk of interaction with positively charged molecules, as is the case of IgG at neutral to slightly alkaline pH (the isoelectric point of IgG ranges from 6.0 to over 9.0) (14). This is reflected in higher binding of IgG to MDA-LDL than to copper-oxidized LDL (61) as a result of the higher level of modification of MDA-LDL. Thus, the need to standardize the assay and correct for nonspecific binding should be obvious. The approaches to carry out such corrections are also quite different (4, 8, 15, 28, 32, 39, 44, 60), but the most popular and least acceptable approach is to correct the results by calculating a difference or ratio between the optical density (OD) values obtained with modified and native LDL (4, 6, 8, 28, 32, 44). Such correction could eliminate the interference of antibodies to native LDL, which to our knowledge have never been proven to exist, while it will fail to correct for nonspecific interactions with modified LDL. The protocols of the few commercially available kits (Alpco Diagnostics [Windham, N.H.] and Kamiya Biomedical Co. [Seattle, Wash.]) do not involve any correction for nonspecific binding.

Few groups give any details about the source of LDL to be used for oxidation and the methods used to control the extent of modification of their oxidized LDL. The same is true for the two commercial kits that we know to be available. As noted by Craig et al. (7) the reproducibility of the data is improved when a pooled LDL is used to prepare oxLDL, a practice that we have also followed in our laboratory. As for controlling the extent of oxidation, few groups report their approach to this

TABLE 2. Comparison of the data obtained in human IgG subclass assay by radial immunodiffusion (RID) and EIA

Assay	Immunoglobulin concn (mg/liter)		
	IgG1	IgG2	IgG3
RID	62 ± 23	6.2 ± 2.5	18 ± 11
EIA	68 ± 40	33 ± 24	18 ± 9
Statistical comparison <sup>a</sup>	NS <sup>b</sup>	$P = 0.038$	NS

<sup>a</sup> Mann-Whitney test.

<sup>b</sup> NS, not significant.

TABLE 3. Comparison of the main features of published assays and commercially available kits for the assay of modified LDL antibodies<sup>a</sup>

Author(s)	Type of assay	Antigen coating	Modification control	Calibrators; units	Quality controls
Virella and others (21, 25, 60)	Competitive EIA for oxLDL total antibody	Copper-oxidized LDL, 7.5 µg/ml in 1-mol/liter bicarbonate buffer, pH 9.6	Continuous monitoring of fluorescence emission (25)	Human serum with known antibody concentration (21); values calculated as mg/liter	Added to each plate
Palinski et al. (39) Hulthe et al. (15)	Direct binding EIA for IgG and IgM antibodies; postcoating of plates to control nonspecific absorption	Copper-oxidized LDL and MDA-LDL, 5 µg/ml in PBS (pH 7.4) containing antioxidants	Electrophoretic mobility in agarose gels	One internal standard; values calculated as ratio unknown (serum minus postcoat)/internal standard (serum minus postcoat)	None mentioned
Erkkilä et al. (8); Maggi et al. (28, 55); Monaco et al. (32)	Direct binding EIA for oxLDL total antibody; native LDL-coated plates to control nonspecific absorption	Copper-oxidized LDL, 2 µg/ml (8), 5 µg/ml (28), MDA-LDL, 5 µg/ml (28, 55), in PBS containing antioxidants, LDL oxidized with peroxidase + hydrogen peroxide (32)	Not mentioned	Values calculated as the ratio between binding to oxLDL and that to native LDL (8; ref. 146 of ref. 28) or as the difference in OD between binding to oxLDL and that to native LDL (55)	Added to each plate (8)
Wu and Lefvert (71)	Direct binding EIA for oxLDL antibodies of different isotypes	Copper-oxidized LDL, 2 µg/ml in 5 mM bicarbonate buffer, pH 9.7	TBARS assay	Values expressed as OD	None mentioned
Wu et al. (72)	Chemiluminescence assay for IgG and IgM antibodies to oxLDL and MDA-LDL	Copper-oxidized LDL and MDA-LDL, 5 µg/ml in TBS containing antioxidants	Not mentioned	Values expressed as relative luminescence units	None mentioned
Salonen et al. (44)	RIA for antibodies to oxLDL and MDA-LDL	Copper-oxidized LDL and MDA-LDL, 5 µg/ml in PBS containing antioxidants	Measurement of MDA-modified lysines in MDA-LDL	Values expressed as the ratio between binding to oxLDL and that to native LDL	None mentioned
Boullier et al. (4); Craig (6)	Direct binding EIA for antibodies to MDA-LDL	MDA-LDL, 5 µg/ml (4), 25 µg/ml (6)	Electrophoretic mobility; TBARS assay	Values expressed both as the ratio and as the difference in OD between binding to oxLDL and that to native LDL	None mentioned
Craig (6)	EIA for antibodies to MDA-LDL	MDA-LDL, 25 µg/ml	TBARS assay		
Alpco Diagnostics; Kamiya Biomedical Co.	EIA for oxLDL antibodies	Copper-oxidized LDL	Not mentioned	Values expressed as mU/ml, based on calibration with a reference serum	High and medium quality control samples

<sup>a</sup> Abbreviations: RIA, radioimmunoassay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; ref., reference.

important issue. Some groups use changes in electrophoretic mobility (4, 15, 39) or thiobarbituric acid-reactive substance (TBARS) assay (4, 6, 72), but both approaches have poor sensitivity and, as such, are of very limited value. Salonen et al. measured MDA-modified lysine residues in MDA-LDL (44), and although this is arguably the most precise way to assess the degree of modification of MDA-LDL, it is not a very easy test

to implement in a clinical laboratory. In our laboratory we have routinely used fluorescence emission to assess the degree of modification of copper-oxidized LDL, and the adoption of this step resulted in improved reproducibility of the assay (25).

The importance of blocking has been underlined by Craig (6) as well as by Naravainen et al. (34). Both conclude that blocking with human serum albumin (0.5 to 1%) decreases

nonspecific binding of IgG to LDL-coated plates. However, different groups continue using different blocking agents, including bovine serum albumin (48, 60) and fetal bovine serum (28)

There are also wide variations in the way that different groups standardize the assays and calculate the results. While the use of quality control samples has become more common, those quality controls are not exchanged by different groups to ensure the reproducibility of the data generated by different assays. As for the calculation of results, there are also considerable variations, from direct measurements of OD (71) or luminescence units (72) to calculations involving a variety of correction factors (15, 34, 39). The two commercial kits for which we have information use calibrators with high antibody concentrations expressed as arbitrary units to calculate unknown concentrations. To our knowledge, only our group has calibrated the assay of oxLDL antibodies with a calibrator with a known absolute concentration of antibody, calculated from serial dilutions of a purified human oxLDL antibody (21). Although the accuracy of the values calculated with our calibrator can be questioned because of the relatively low affinity of modified LDL antibodies, if such standards, relatively easy to obtain, were widely used, the data obtained by different groups would be easier to compare.

Assays of autoantibodies reacting with other modified lipoproteins, such as AGE-LDL, have been developed by a few groups (54, 68), and there are less experience and fewer data to assess their degree of reproducibility.

#### FREE VERSUS COMPLEXED MODIFIED LDL ANTIBODIES

Irrespective of all and every effort that may be applied to the development of precise assays for modified LDL antibodies, there is an intrinsic factor that contributes to their inaccuracy that cannot be easily eliminated. If the average avidity of circulating lipoprotein autoantibodies is sufficiently high and antigen is present in circulation, soluble IC are formed. Several groups have established the presence of soluble LDL-IC in circulation (5, 9, 24, 30, 31, 37), and we and others have also shown that the avidity of complexed antibodies is significantly higher than that of free, circulating antibodies (1). There is an inverse correlation between concentrations of circulating LDL-IC and serum oxLDL antibody concentrations (24) showing that, in the presence of soluble IC, the assays for oxLDL antibodies underestimate the concentration of total antibodies in circulation.

Soluble LDL-IC have been detected and characterized in two basic ways: capture EIAs and precipitation with polyethylene glycol (PEG). Capture EIA usually involves the immobilization of an antibody to LDL (modified or not) and the detection of cocaptured immunoglobulins with enzyme-labeled antibodies (4, 72). It is the simplest approach, allows the identification of antibody isotypes involved in IC formation, and is suitable for large-scale screening. On the negative side, it does not properly differentiate between true LDL-IC and nonspecifically adsorbed IC. It is also a qualitative test, and there is no easy way to calibrate the results. PEG precipitation is certainly nonspecific, and the assay of PEG-precipitated IC, the basis of some of the oldest assays for circulating IC (58, 59), is totally

nonspecific from the antigen point of view. However, if the method is properly standardized so that the antigen of interest is not precipitated in its free form, antigen detection in PEG precipitates becomes a specific measurement of specific antigen-antibody complexes (24, 37). Furthermore, PEG-based assays can be quantitative and are easily scaled up to become preparative, allowing then the characterization of coprecipitated antigens and antibodies. (30, 31, 68). On the negative side, PEG-based assays are tedious, require well-trained personnel for their proper execution, and are not easy to adapt to large-scale screening protocols.

The nature of the antibodies involved in soluble LDL-IC formation has been investigated by both approaches. Capture assays have shown that both IgG and IgM can be cocaptured with LDL, suggesting that both immunoglobulins are involved in IC formation (53, 72) However, as noted above, any attempts at trying to determine the preponderance of either type of antibody by capture assays are doomed by the impossibility of calibrating the assay.

In PEG precipitation assays, IgM and IgG can be detected in the precipitate, but IgM can be nonspecifically precipitated by the concentrations of PEG used for isolation of IC, so the direct assay of IgM in PEG precipitates is of limited value. By EIA it is possible to measure IgG and IgM antibodies to modified LDL in resuspended PEG precipitates (53, 72), but the assays are grossly inaccurate because of the interference of IC in binding assays.

By using slightly scaled-up versions of the PEG precipitation protocol used for the screening of circulating IC, it is possible to obtain enough material to perform more detailed analytical and quantitative studies. It is important that in order to define and measure the antibodies involved in LDL-IC formation it is essential to separate the IC into its constituent antigens and antibodies. This can be done with relative ease by taking advantage of the moderate avidity of modified LDL antibodies and of the high-affinity interaction between streptococcal protein G and IgG. Thus, affinity chromatography in immobilized protein G with a modified elution protocol allows the separation of IgG without LDL contamination (1). Such IgG can be shown to react with oxLDL and AGE-LDL and, in the case of oxLDL antibodies, to have a significantly higher avidity than "free" LDL antibodies purified directly from serum samples (1). On the other hand, this protocol also allows the isolation of ApoB-containing lipoproteins from the protein G washout, thus allowing a direct glimpse into the modifications of LDL present in molecules involved in IC formation (68). What this protocol does not allow is the easy isolation of IgM antibodies, and therefore the question of whether IgM antibodies predominate over IgG antibodies cannot be resolved by any of the approaches currently used for LDL-IC assay.

#### CLINICAL SIGNIFICANCE OF MODIFIED LDL ANTIBODIES

The controversy surrounding the clinical significance of modified LDL antibodies results from a variety of different factors. As mentioned, methodology inaccuracies, the interference of soluble IC, and the lack of standardization of LDL antibody assays are major confounding factors. For as many carefully executed assays that seem to demonstrate that high

levels of oxLDL antibodies are indicative of atherosclerotic vascular disease, progression of carotid atherosclerosis, or the risk for the future development of myocardial infarction (2, 8, 22, 28, 39, 44, 53), there are conflicting studies showing either no correlation or an inverse correlation (4, 9, 16, 17, 23, 24, 36, 55, 56, 60, 72).

Given that the antibodies most likely to be proinflammatory are those of higher avidity, involved in soluble IC formation (1), it seems logical to propose that the assay of IC-associated antibodies is more likely to provide clinically relevant information, and the data accumulated by our group certainly support this position (1, 24, 36). Of particular significance was the observation made in a prospective study involving 98 patients with type 1 diabetes, half of whom had developed coronary heart disease (CHD) during an 8-year follow-up period and half of whom had not developed CHD over the same period. In this study we demonstrated that the levels of LDL-IC were significantly higher in the group of patients that developed CHD than in the group that did not (24, 36). In another study involving over 1,000 patients with type 1 diabetes we have also observed a significant direct correlation between high levels of LDL-IC and intima-media carotid thickness (unpublished results).

Strong support for the pathogenic role of IC can also be found in *in vitro* studies with IC prepared either with rabbit LDL antibodies (11, 62) or with isolated human oxLDL antibodies (66) as well as in *ex vivo* studies performed with LDL-containing IC isolated from human sera by PEG precipitation (30, 31, 52).

In contrast, the postulated "protective" role of modified LDL antibodies (3, 41, 46, 70, 75), primarily based on observations carried out with experimental animals, fraught with contradictory data (26), has very little support from human studies. The problems inherent to the assay of serum ("free") modified LDL antibodies very much discredit any observed trends favoring a protective or a pathogenic role of those antibodies. It is also important to note that there are very few *in vitro* data supporting such a protective role, other than some observations performed with IgM antibodies cloned from experimental animals (46).

Some groups have widely publicized this possible protective role of IgM antibodies, the basis for the "immunomodulating" role recently proposed for modified LDL antibodies. Several considerations need to be made in this regard. First and foremost is the fact that the predominant immunoglobulin isotypes in affinity chromatography-purified modified LDL antibodies are the unquestionably proinflammatory IgG1 and IgG3 subclasses of IgG (29, 63). It can be argued that IgM antibodies could predominate in LDL-IC. The previously discussed limitations of the assays for LDL-IC when it comes to determining the concentration of IgM antibodies involved in the formation of circulating IC limit the validity of any conclusion about this particular issue. However, the pathogenic IC are more likely to be formed in the atheromatous plaque and surrounding tissues, and IgM antibodies are highly unlikely to predominate on the extravascular compartment given their large molecular size and predominantly intravascular distribution (64).

In conclusion, it is likely that the controversy surrounding the clinical significance of modified LDL antibodies may continue for a few years, but at this point the balance of evidence

suggests that those antibodies are potentially pathogenic and that their measurement could add significant information to the evaluation of cardiovascular risk.

#### CAN THE ASSAYS OF FREE OR BOUND MODIFIED LDL ANTIBODIES BE USED FOR ROUTINE EVALUATION OF ATHEROSCLEROSIS RISK?

A variety of assays have been developed for the assay of both free and IC-bound antibodies to modified forms of LDL. The assays suffer from several flaws, some of which could be corrected—e.g., standardization and calibration—and some of which—e.g., the interference of IC—are more difficult to correct with the present assays. Furthermore, there is a need to reevaluate the principles on which modified antibody assays are based, having the goals of the assay in mind. If the main goal is to determine whether modified LDL antibodies represent a risk factor for the development of atherosclerosis, the assay needs to consider that the main risk derives from the synthesis of antibodies with avidity sufficiently high as to form stable, proinflammatory IC. Thus, the relevant assays need to be designed to detect and measure such antibodies. This can certainly be done with assays based on PEG precipitation, but such assays have significant limitations when it comes to their use in large-scale clinical screening studies. New approaches to the assay of both free and complexed antibodies based on both their specificity and avidity are urgently needed. On the other hand, if the main goal is to assess the presence of modified LDL in circulation, assays specific for different forms of modified LDL would be superior. Either way, a greater spirit of cooperation between groups involved in this research would allow a faster development of generally accepted methodology, properly controlled and standardized. This would certainly allow faster progress in the evaluation of the different approaches for the measurement of the autoimmune response to modified forms of LDL.

#### CONCLUSIONS

The immunogenicity of modified lipoproteins has attracted considerable attention. Antibodies to oxLDL and MDA-LDL have been purified and characterized, and their proinflammatory potential has been established both because of the predominance of IgG1 and IgG3 immunoglobulins in purified antibody preparations (29, 63) and because of the ability of LDL-IC to induce macrophage activation and foam cell formation (10, 11, 30, 62, 66). Much of the confusion surrounding the clinical significance of modified LDL antibodies results from flaws in the assays, clinical studies with small populations, and lack of understanding of the interference of antigen-antibody complexes in serum antibody assays. In reality, the concentrations of free circulating antibody, even if accurately measured, are unlikely to have much clinical significance. The proinflammatory properties of IC are primarily related to antibody isotype and avidity, neither of which is estimated with the conventional EIAs for serum antibody and circulating IC. Of the currently available assays, those based on PEG precipitation are the only ones adaptable to the measurement of IgG-complexed antibodies, their IgG isotype distribution, and avidity. Until newer and less laborious assays are developed,

the PEG precipitation-based assays are the best suited for the investigation of the pathogenic significance of modified LDL antibodies. But there is a clear need to develop alternative approaches, better suited for large-scale studies and avoiding the known pitfalls of the present assays.

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