# Definition of the immunogenic forms of modified human LDL recognized by human autoantibodies and by rabbit hyperimmune antibodies

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**Abstract** **Humans and laboratory animals recognize human modified LDL as immunogenic. Immune complexes (ICs) isolated from human sera contain malondialdehyde-modified LDL (MDA-LDL) and $N^{\varepsilon}$(carboxymethyl)lysine-modified LDL (CML-LDL) as well as antibodies reacting with MDA-LDL, copper-oxidized LDL (OxLDL), CML-LDL, and advanced glycosylation end product (AGE)-modified LDL. OxLDL and AGE-LDL antibodies isolated from human sera recognize the same LDL modifications and do not react with modified non-LDL proteins. Rabbit antibodies have different reactivity patterns: MDA-LDL antibodies react strongly with MDA-LDL and MDA-BSA but weakly with OxLDL; OxLDL antibodies react strongly with OxLDL and weakly with MDA-LDL; CML-LDL antibodies react with CML-LDL > CML-BSA > AGE-LDL > OxLDL; AGE-LDL antibodies react strongly with AGE-LDL, react weakly with OxLDL, and do not react with CML-LDL. Thus, human and rabbit antibodies seem to recognize different epitopes. Capture assays carried out with all rabbit antibodies showed binding of apolipoprotein B-rich lipoproteins isolated from ICs, suggesting that laboratory-generated epitopes are expressed by in vivo-modified LDL, although they are not necessarily recognized by the human immune system. Thus, the definition of immunogenic forms of modified LDL eliciting human autoimmune responses requires the isolation and characterization of autoantibodies and modified LDL from human samples, whereas rabbit antibodies can be used to detect in vivo-modified human LDL.**—Virella, G., S. R. Thorpe, N. L. Alderson, M. B. Derrick, C. Chassereau, J. M. Rhett, and M. F. Lopes-Virella. **Definition of the immunogenic forms of modified human LDL recognized by human autoantibodies and by rabbit hyperimmune antibodies.** ***J. Lipid Res.*** **2004.** 45: **1859–1867.**

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The involvement of immune mechanisms in the pathogenesis of atherosclerosis has received considerable attention in recent years (1, 2). Both cell-mediated and humoral mechanisms have been proposed as playing a role in the initiation or perpetuation of vascular inflammation (2). Modified lipoproteins are among the antigens that have been proposed to elicit immune responses relevant to the pathogenesis of atherosclerosis (2). This hypothesis is based on a large body of data. The immunogenicity of modified lipoproteins, first reported by Steinbrecher et al. (3), was well documented in studies involving the immunization of laboratory animals with in vitro-modified lipoproteins. Human autoantibodies reacting with copper-oxidized LDL (OxLDL) have also been well characterized (4–6).

LDL oxidation affects both the lipid and protein components of LDL. Reactive aldehyde products formed during the oxidation of polyunsaturated fatty acids, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), are capable of attaching covalently to the ε-amino groups of lysine residues of apolipoprotein B (apoB) (7–9). These modifications are present in OxLDL as well as in LDL isolated from atherosclerotic plaques, which reacts with monoclonal antibodies produced in guinea pigs against MDA and HNE-lysine (9, 10).

Detailed investigations have also been carried out with advanced glycosylation end product (AGE)-modified 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 col-

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lectively known as "advanced glycosylation end products" (11). LDL, like most plasma proteins, is susceptible to AGE modification (12). AGE-modified proteins are immunogenic (13), and the corresponding antibodies raised in laboratory animals have been used for the detection of AGE-modified proteins in serum (14) and tissues (14, 15). In humans, AGE-LDL antibodies have been purified from sera with high antibody titers and characterized as far as isotype distribution, avidity, and specificity (16). Of the two best characterized modifications of AGE proteins, $N^{\varepsilon}$(carboxymethyl)lysine (CML) and $N^{\varepsilon}$(carboxyethyl)lysine (CEL), CML seems to be the most immunogenic.

The comparison of data reported in the literature, based on animal immunization studies, with data generated from the study of purified human and mouse OxLDL autoantibodies suggests that MDA-lysine is a most significant epitope (17, 18), but significant differences seem to exist between the specificity of human and hyperimmune animal antibodies to modified lipoproteins. For example, Steinbrecher et al. (3) showed that immune guinea pig antibodies to carbamylated, acetylated, or ethylated autologous LDL reacted with the corresponding modified lysine residues and that the reactivity extended to other modified non-LDL proteins. Similarly, it has been reported that antibodies raised in rabbits against one AGE-modified protein react with other AGE-modified proteins (19). In contrast, affinity chromatography-purified human AGE-LDL antibodies react primarily with AGE-LDL, cross-react strongly with CML-LDL and OxLDL, but show very little to minimal reactivity with CML-human serum albumin (HSA) (16). These discrepancies raise important questions about species differences in epitope recognition and about the nature of the epitopes recognized by spontaneously formed human autoantibodies and antibodies induced in animals by immunization with chemically modified LDL. To resolve some of these questions, we have characterized the nature of the predominant modifications in human apoB-rich lipoproteins obtained from antigen-antibody complexes isolated from human sera and undertaken a comparative study of the reactivity of purified human autoantibodies to OxLDL and AGE-LDL with antibodies obtained in rabbits immunized with different modifications of human LDL.

## METHODS

### Human autoantibodies

Human autoantibodies to OxLDL and AGE-LDL were purified from serum and from polyethylene glycol (PEG)-precipitated human immune complexes (ICs) as reported in previous publications (16, 20) using the same patient population. Informed consent as approved by the Institutional Review Board for Human Research of every center involved in sample collection was obtained from each subject included in this study. The serum samples used in this study were kept at −70°C until our studies were performed. Serum antibodies were isolated by affinity chromatography on columns of immobilized modified human LDL (OxLDL or AGE-LDL) as previously reported (5, 16), with slight modifications in the elution step for AGE-LDL antibodies. After loading the AGE-LDL column and incubating it overnight at 4°C, the unbound protein was washed off with 0.01 M $NaHCO_3$ buffer, pH 8.3, containing 0.005 M NaCl. The bound antibody was eluted with 0.1 M $NaHCO_3$ buffer, pH 8.0, containing 1.5 M NaCl. The isolation of lipoprotein-free IgG from PEG-precipitated ICs was achieved by affinity chromatography on protein G-Sepharose (Amersham-Pharmacia, Piscataway, NJ) using previously reported conditions (16, 20).

### Preparation of modified proteins

OxLDL was prepared according to our modification (21) of the protocol described by Steinbrecher (8). Human LDL was diluted in PBS to a concentration of 1,500 mg/l apoB and incubated with 40 μmol/l $CuCl_2$. The degree of oxidation was monitored continuously by fluorescence emission using a fluorescence spectrophotometer (SLM-AMINCO® Series 2; Spectronic Instruments, Rochester, NY). LDL oxidation was stopped 4–6 h after the fluorescence values reached the peak (≥1.1 fluorescence units).

AGE-LDL was prepared by a modification of the method described by Schmidt et al. (22). Freshly isolated LDL (1.2 mg/ml) was sterilized by passage through a 0.2 μm filter, added to 150 mM glucose-6-phosphate in 200 mM phosphate buffer, pH 7.8, containing 40 μM butyl-hydroxytoluene (BHT) and 540 μM EDTA, filter-sterilized a second time, and incubated for 8 weeks at 37°C. At the end of the incubation, the LDL solution was dialyzed for 24 h against three changes of 4 liters of 0.15 M NaCl, 0.3 mM EDTA, pH 8.0. Our AGE-LDL modifications contained CML and CEL (**Table 1**). The MDA-lysine content of AGE-LDL was usually below the detection limit, but occasionally small amounts of MDA-lysine were detected.

MDA modification of proteins was performed according to Haberland, Fogelman, and Edwards (23) by incubating equal volumes of freshly isolated human LDL or BSA (Cohn fraction V; Sigma, St. Louis, MO) and 0.2 M MDA for 3 h at 37°C, followed by extensive dialysis against 0.15 M NaCl with 0.3 mM EDTA, pH 8.0.

CML-modified proteins were prepared by incubation of the protein with glyoxylic acid and $NaBH_3CN$ in phosphate buffer at 37°C, as described previously for the preparation of CML-BSA (24). In each case, control protein was incubated under the same conditions but without glyoxylic acid.

### Analysis of LDL modifications

Analysis of modified lipoproteins for their content of CML, CEL, and the advanced lipoxidation end products (ALEs) MDA-lysine and HNE-lysine was carried out by selected ion monitoring (SIM)-GC-MS as described by Requena et al. (11). Briefly, these four AGEs/ALEs were measured simultaneously in modified LDLs (AGE-LDL, OxLDL) and in LDL isolated from human se-

TABLE 1. Characterization of the forms of modified LDL used in rabbit immunizations

| Modified LDL | CML Content | CEL Content | MDA Content |
|---|---|---|---|
| OxLDL | 0.8 (0.08%) | 0.25 (0.025%) | 6.75 (0.6%) |
| MDA-LDL | n.d.[a] | n.d. | 245 (25%) |
| MDA-BSA | n.d. | n.d. | 168 (17%) |
| AGE-LDL | 4.6 (0.5%) | 0.45 (0.05%) | n.d.–0.5 (0.05%) |
| CML-LDL | 300–500 (30–50%) | n.d. | n.d. |
| CML-BSA | 370 (37%) | n.d. | n.d. |

AGE, advanced glycosylation end product; CEL, $N^{\varepsilon}$(carboxyethyl)lysine; CML, $N^{\varepsilon}$(carboxymethyl)lysine; MDA, malondialdehyde; OxLDL, copper-oxidized LDL. All values are expressed in mmol/mol lysine, with the percentage of modified lysines in parentheses.

[a] n.d., not detected.

rum (0.5–1.0 mg of protein) after borohydride reduction of the protein. The reduced sample was dialyzed, dried, delipidated [chloroform-methanol (2:1) containing 0.02% BHT], and, after the addition of heavy labeled internal standards, hydrolyzed at 110°C in 6 N HCl for 18 h. The hydrolysate was dried, passed over a 1 ml solid phase extraction $C_{18}$-column (Sep-Pak; Waters, Milford, MA), and dried again, and the amino acids were derivatized as their trifluoroacetyl methyl esters for analysis by SIM-GC-MS (11, 14, 25). Quantification was performed by isotope dilution, and all modified amino acids are expressed as a ratio to the parent amino acid, lysine.

The degree of modification of MDA-LDL was estimated by the thiobarbituric acid reactive substances assay (26) using MDA as a standard. The extent of modification of CML-LDL and CML-albumins was measured by amino acid analysis (24).

### Rabbit antibodies

Antibodies to modified LDL were obtained by immunization of New Zealand White female rabbits with different modifications of human LDL (AGE-LDL, CML-LDL, OxLDL, and MDA-LDL). The degree and nature of modification of the lipoproteins used for immunization are summarized in Table 1. Two to four rabbits were immunized per each modified human LDL. The lipoproteins were diluted in sterile saline to 2.5 mg/ml. Each LDL preparation was emulsified with an equal volume of complete Freund's adjuvant immediately before intramuscular inoculation. Each rabbit was immunized with 1 ml of emulsion. Two weeks later, the rabbits were inoculated a second time with an identical amount of modified LDL emulsified with incomplete Freund's adjuvant by the same route. A third inoculation was administered 2 weeks later using antigen diluted in saline and injected subcutaneously in four divided doses placed in the dorsal area. Two weeks after the third injection, the rabbits were bled and euthanized. The immunization and bleeding protocols were approved by the university's Institutional Animal Care and Use Committee.

The sera obtained at exsanguination, containing a mixture of antibodies to apoB and to the modified LDL, were fractionated on a protein G-Sepharose column. The eluted IgG fraction was then absorbed on a column of native LDL-Sepharose. The washout from that column contained a mixture of IgG antibodies to modified LDL and irrelevant IgG. The pattern of reactivity with the different modified LDL preparations and controls was consistent for each set of rabbits immunized with a given modified LDL. However, there were variations in antibody titer from rabbit to rabbit, and the antiserum with a higher titer was selected for further characterization.

An alternative protocol was designed to determine whether immobilized OxLDL contained CML epitopes. In this case, the IgG fraction from a CML-LDL antiserum was absorbed first on an immobilized native LDL column and the washout was fractionated on an OxLDL column. After washing the OxLDL column extensively with 0.01 M sodium bicarbonate buffer, pH 8.3, bound antibodies were eluted with 0.1 M sodium bicarbonate buffer containing 0.5 M NaCl, pH 8.3, and tested for reactivity with CML-LDL.

### Determination of antibody specificity

The specificity of purified human and rabbit antibodies was determined through a variety of approaches. The basic pattern of reactivity for both human and rabbit antibodies was established by competition studies, in which different antigens were preincubated with a given antibody to determine their ability to inhibit binding with immobilized antigens. The inhibition studies were designed as modifications of our previously reported competitive enzymoimmunoassay (EIA) for oxidized LDL antibodies (27). The EIA plates were coated with 0.75 μg/well of protein, including modified forms of LDL (OxLDL, AGE-LDL, MDA-LDL, or CML-LDL), other modified proteins (MDA-HSA or CML-BSA), and native LDL, according to the experiment being performed. In all competition studies, the proteins preincubated with the different antibodies were adjusted to a final concentration of 200 μg/ml.

In addition, capture assays were carried out in experiments designed to complement the competition studies. These assays were carried out with purified rabbit IgG antibodies of different specificities absorbed to the plates at a 1:10 dilution. After washing off the unabsorbed antibodies, the plates were blocked with 5% BSA. Serial dilutions of modified LDL preparations were added to the plates, and peroxidase-conjugated goat anti-rabbit apoB (1:1,000 dilution; Academy Bio-Medical, Houston, TX) was used to detect bound LDL. Capture assays were also used to determine whether the rabbit antibodies had the potential to capture in vivo-modified LDL, obtained as described below.

### Comparison of the degree of modification of soluble apoB-rich lipoproteins versus apoB-rich lipoproteins precipitated with 4% PEG

Fractionation of human sera with 4% PEG was performed as previously described (16). ApoB-containing lipoproteins were isolated from the supernatant and from the resuspended PEG precipitates. The supernatants were fractionated directly on heparin-agarose columns (Sigma-Aldrich Corp., St. Louis, MO) that retain apoB-containing lipoproteins. The PEG precipitates were first submitted to affinity chromatography on protein G-Sepharose, and the washout, containing all precipitated proteins other than IgG, was then fractionated on heparin-agarose columns. The lipoprotein-containing samples were pooled and dialyzed against saline containing 0.3 mM EDTA, pH 8.0, and analyzed for modifications as described above.

## RESULTS

### Analysis of the degree of modification of soluble and IC-associated apoB-rich lipoproteins

The apoB-rich lipoproteins (LDL and lipoprotein [a]) fractionated from PEG precipitates of human sera were significantly more modified than the apoB-rich lipoproteins remaining in solution (**Fig. 1**). CML was detected in both lipoprotein fractions, but in significantly higher concentrations in precipitated lipoproteins ($P < 0.0001$). The concentration of CEL was also significantly higher in precipitated lipoproteins ($P = 0.0011$), although the absolute concentration in soluble and precipitated fractions was much lower than for CML, and the values showed a greater overlap. However, for both modified amino acids there was an ~3-fold enrichment in the precipitated compared with the soluble proteins. The difference was even more marked in the case of MDA-lysine, which was rarely detected in soluble lipoproteins (6 of 37 samples), whereas it was always detected in precipitated lipoproteins ($P < 0.0001$). HNE-lysine was analyzed but not detected.

### Reactivity of IgG isolated from PEG-precipitated human ICs

The reactivity of IgG isolated from PEG-precipitated ICs was determined by inhibition studies using immobilized

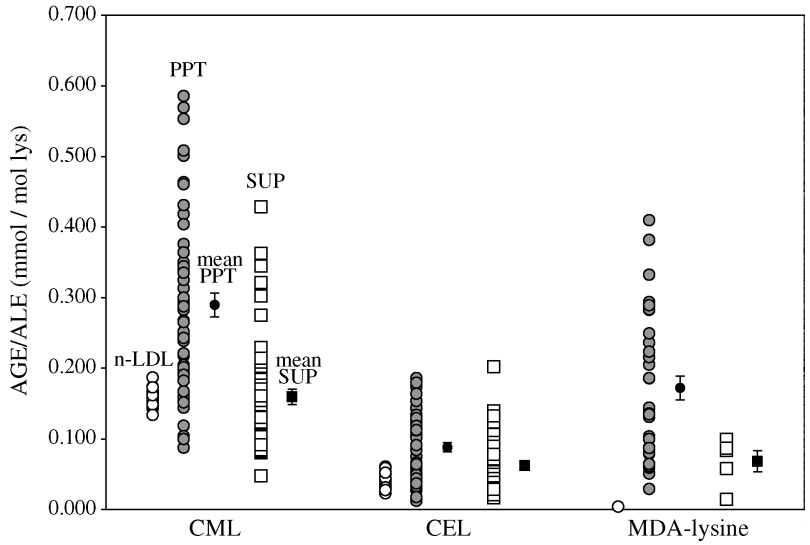


**Fig. 1.** Comparison of the degree of modification of soluble (SUP) and insoluble (PPT) apolipoprotein B (apoB)-rich lipoproteins separated by affinity chromatography on heparin-agarose after 4% polyethylene glycol (PEG) fractionation (n = 53 for precipitates and 52 for supernatants). The extent of modification of native LDL (nLDL; n = 19) is shown for reference. Bars show means ± SEM. 4-Hydroxynonenal-lysine was analyzed but not detected. AGE, advanced glycosylation end product; ALE, advanced lipoxidation end product; CEL, $N^{\varepsilon}$(carboxyethyl)lysine; CML, $N^{\varepsilon}$(carboxymethyl)lysine; lys, lysine; MDA, malondialdehyde.

OxLDL and AGE-LDL as substrates. When immobilized OxLDL was used as the substrate, OxLDL and MDA-LDL were the most effective competitors (**Fig. 2A**). Native LDL and MDA-BSA did not compete for the binding with OxLDL. When immobilized AGE-LDL was used as the substrate, the overall degree of reactivity was lower (highest optical density at 414 nm was 0.250 vs. a highest optical density at 414 nm of 0.680 measured in OxLDL-coated plates), and, as expected, AGE-LDL and CML-LDL competed for the binding with immobilized AGE-LDL. Native LDL and CML-BSA showed weak degrees of competition with immobilized AGE-LDL (Fig. 2B).

### Reactivity of purified human antibodies and rabbit antibodies to MDA-LDL and OxLDL

Human antibodies to oxidized LDL isolated from serum showed primary reactivity with OxLDL and MDA-LDL and a low degree of cross-reactivity with MDA-BSA (**Table 2**). Neither AGE-LDL nor native LDL reacted significantly with human OxLDL antibodies.

In contrast, in competition assays rabbit OxLDL antibodies not only recognized OxLDL and MDA-LDL but also reacted with AGE-LDL, CML-LDL, and native LDL (Table 2). This broad reactivity of rabbit OxLDL antibodies is limited to LDL and its modifications, because prein-

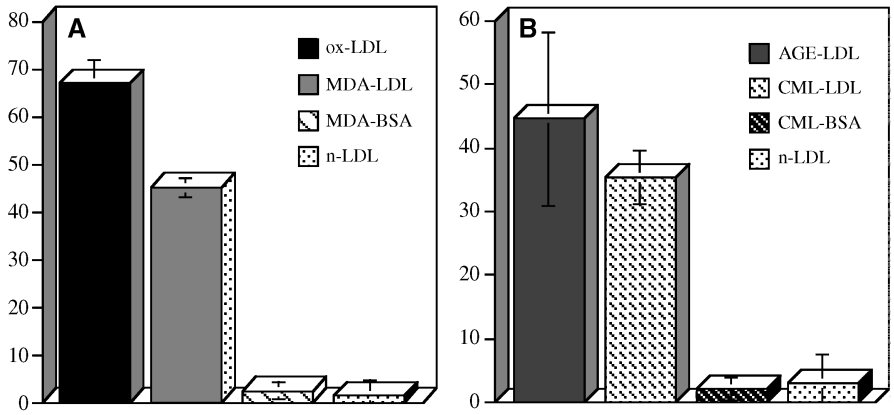


**Fig. 2.** Reactivity of IgG immunoglobulins purified from 4.0% PEG precipitates with human copper-oxidized LDL (OxLDL; A) and human AGE-LDL (B). Purified IgG aliquots were incubated with equal concentrations of different preparations of modified LDL and modified BSA as well as with native LDL. The data are expressed as means ± SEM percentage reduction in reactivity with immobilized OxLDL (A) or immobilized AGE-LDL (B) after absorption with the indicated modified LDL: OxLDL, MDA-LDL, MDA-BSA, AGE-LDL, CML-LDL, CML-BSA, and native LDL (n = 3 in all conditions depicted in A and B).

TABLE 2. Reactivity of human and rabbit OxLDL antibodies with different types of modified lipoproteins determined by competitive ELISA

| Antibodies | Antigens Used for Absorption | | | | | | |
|---|---|---|---|---|---|---|---|
| | OxLDL | MDA-LDL | AGE-LDL | CML-LDL | MDA-BSA | CML-BSA | Native LDL |
| Human OxLDL | 76.2 ± 0.9% (n = 52) | 75.9 ± 1.5% (n = 52) | 5.1 ± 3.6% (n = 13) | Not reactive (n = 6) | 16.5 ± 1.9% (n = 6) | 4.5 ± 7.0% (n = 4) | 6.1 ± 0.6% (n = 52) |
| Rabbit OxLDL | 96.3 ± 0.3% (n = 6) | 40.0 ± 4.2% (n = 6) | 59.3 ± 4.1% (n = 6) | 33.0 ± 3.5% (n = 5) | Not reactive (n = 5) | Not reactive (n = 5) | 27.7 ± 3.7% (n = 6) |
| Rabbit MDA-LDL | 25.4 ± 1.0% (n = 4) | 97.4 ± 1.7% (n = 4) | 0.5 ± 5.8% (n = 4) | 0.2 ± 4.0% (n = 4) | 88.0 ± 2.4% (n = 4) | 1.0 ± 5.2% (n = 4) | 1.6 ± 3.8% (n = 4) |

Values shown are expressed as percentage reduction ± SEM in reactivity with immobilized OxLDL after incubation with the different antibodies indicated.

cubation with MDA-BSA did not affect their reactivity with immobilized OxLDL. The cross-reactivity with native LDL, MDA-LDL, AGE-LDL, and CML-LDL could be explained by the presence of residual unabsorbed antibodies to apoB. However, a repeated passage through a column of immobilized native LDL had no effect on the reactivity pattern. The absorbing efficiency of the native LDL column was verified by the elution of highly reactive apoB antibodies after the first passage of the OxLDL antibody. No additional antibodies were recovered after the second passage. On the other hand, in capture assays the reactivity of rabbit OxLDL antibodies was limited to OxLDL (**Fig. 3**).

Human MDA-LDL is highly immunogenic in rabbits, and the resulting antibodies recognize an MDA-associated epitope that is shared by MDA-LDL and MDA-BSA (Table 2). In contrast, the reactivity of MDA-LDL antibody with OxLDL is significantly lower, and the antibody does not recognize AGE-LDL and native LDL. Capture assays paralleled the data obtained with absorption studies, showing that MDA-LDL was preferentially captured, and from the other tested proteins only OxLDL was captured in significant concentrations (**Fig. 4**).

### Reactivity of purified human antibodies and rabbit antibodies to AGE-LDL and CML-LDL

Human circulating AGE-LDL antibodies reacted primarily with AGE-LDL, but they also reacted with OxLDL and CML-LDL and to a lesser extent with native LDL (**Table 3**). The epitope recognized by these human antibodies is LDL-specific, because their reactivity was not affected by absorption with CML-BSA.

Rabbit AGE-LDL antibodies reacted strongly with AGE-LDL and very weakly with OxLDL when tested by absorption (Table 3). In capture assays, they reacted strongly with AGE-LDL and also captured OxLDL at high protein concentrations but did not react with CML-LDL (**Fig. 5**). The reactivity with OxLDL relative to the reactivity with AGE-LDL varied between 2.4% at a protein concentration of 0.04 μg/ml and 23% at a protein concentration of 1.25 μg/ml.

The reactivity of rabbit antibodies raised against human CML-LDL was studied in a variety of ways. Competition studies were carried out with two immobilized substrates, the CML-LDL used for immunization and human AGE-LDL (Table 3). Using immobilized CML-LDL, the antibodies reacted strongly with CML-LDL and CML-BSA, moderately with AGE-LDL, and weakly with OxLDL. However, when AGE-LDL was used as the substrate, the reactivity of AGE-LDL and OxLDL increased by ~2-fold. As previously reported, OxLDL contains MDA-lysine, CML-lysine, and HNE-lysine (5), raising the possibility that the CML-LDL antibodies could recognize CML-lysine epitopes in OxLDL. To clarify this issue, the IgG fraction from a rabbit CML-LDL antibody was first absorbed with immobilized native LDL to eliminate apoB antibodies and then

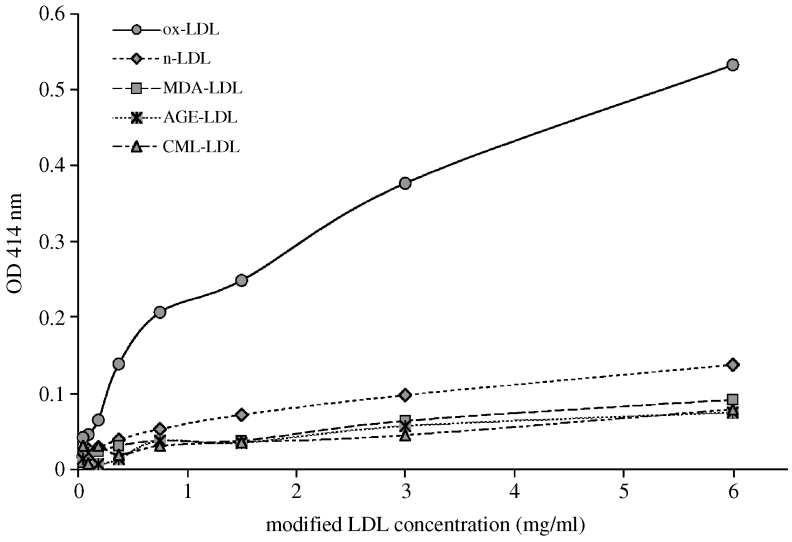


**Fig. 3.** Capture assay with immobilized rabbit OxLDL antibody. The plot represents the optical density (OD) obtained after incubating the different modified LDL and native LDL (nLDL) preparations at variable concentrations with the immobilized antibody. Peroxidase-labeled anti-human apoB was used to detect the captured LDL.

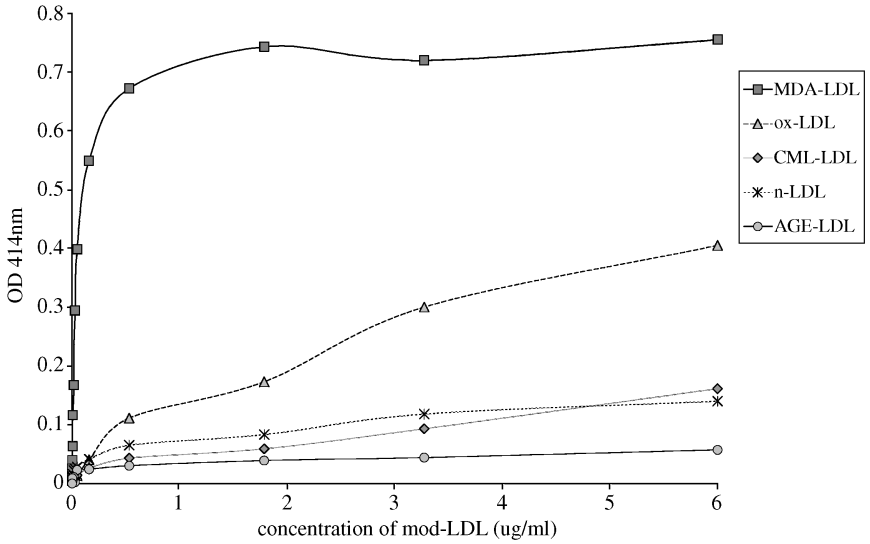


**Fig. 4.** Capture assay with immobilized rabbit MDA-LDL antibody. The plot represents the OD at 414 nm obtained after incubating the different modified LDL (mod-LDL) and native LDL (nLDL) preparations at variable concentrations with the immobilized antibody. Peroxidase-labeled anti-human apoB was used to detect the captured LDL.

fractionated on a column of immobilized OxLDL. The antibody peak eluted from the OxLDL column was tested in a direct binding assay against different LDL modifications as well as native LDL and was found to react strongly and exclusively with CML-LDL.

Capture assays confirmed the exquisite specificity of rabbit anti-CML antibodies for the CML-LDL preparation used as an immunogen (**Fig. 6**, inset). At relatively high concentrations, AGE-LDL and OxLDL were also captured (Fig. 6).

Capture assays carried out with identical concentrations of soluble and IC-bound apoB-rich lipoproteins (5 μg/ml) isolated from human sera showed a significantly higher capture of IC-bound apoB-rich lipoproteins with the four different rabbit antibodies to modified LDL used in this study (**Table 4**).

## DISCUSSION

The reported experiments were carried out with two basic objectives: to define the immunogenic modifications of human LDL eliciting autoantibody formation, and to determine whether in vitro-modified LDL could be used to immunize rabbits and obtain antibodies that could be used in capture assays of in vivo-modified lipoproteins.

To define the immunogenic modifications of LDL that elicit spontaneous autoimmune responses in humans, we characterized the modified forms of LDL and the antibodies purified from isolated ICs. Our data indicate that a minimum of two antigen-antibody systems are involved in the formation of modified LDL-ICs. The significant enrichment of PEG-precipitated ICs in MDA-LDL and the detection of antibodies reacting with both OxLDL, which contains significant concentrations of MDA (5), and MDA-LDL indicate that the MDA modification of LDL is recognized by the human immune system. The equally significant enrichment of PEG precipitates in CML-LDL and the detection of antibodies reacting with AGE-modified LDL, which contains significant amounts of CML-LDL (16), and with CML-LDL indicate that CML-LDL is immunogenic for humans.

The pattern of reactivity of OxLDL and AGE-LDL antibodies isolated from serum was similar to that of antibod-

TABLE 3. Reactivity of human and rabbit AGE-LDL antibodies and rabbit CML-LDL antibodies with different types of modified lipoproteins determined by competitive ELISA

| Antibodies | Antigen in Plate Coat | Antigens Used for Absorption | | | | |
|---|---|---|---|---|---|---|
| | | OxLDL | AGE-LDL | CML-LDL | CML-BSA | Native LDL |
| Human AGE-LDL | AGE-LDL | 21.7 ± 6.0% (n = 9) | 46.8 ± 5.8% (n = 9) | 16.0 ± 1.9% (n = 9) | 2.0 ± 2.7% (n = 9) | 13 ± 2.5% (n = 9) |
| Rabbit AGE-LDL | AGE-LDL | 7.9 ± 1.1% (n = 5) | 97.3 ± 0.3% (n = 5) | 5.8 ± 1.0% (n = 5) | 2.4 ± 0.6% (n = 4) | 3.5 ± 1.5% (n = 5) |
| Rabbit CML-LDL | CML-LDL | 17.9 ± 0.7% (n = 4) | 46.7 ± 2.7% (n = 4) | 98.5 ± 0.1% (n = 4) | 72.3 ± 1% (n = 4) | 1.7 ± 0.8% (n = 4) |
| Rabbit CML-LDL | AGE-LDL | 41.4 ± 0.14% (n = 4) | 99.0 ± 0.14% (n = 4) | 99.9 ± 0.08% (n = 4) | 92.9 ± 0.06% (n = 4) | 4.3 ± 0.48% (n = 4) |

Values shown are expressed as percentage reduction ± SEM in reactivity with the antigen coated on the enzymoimmunoassay plate after incubation with the different antibodies indicated.

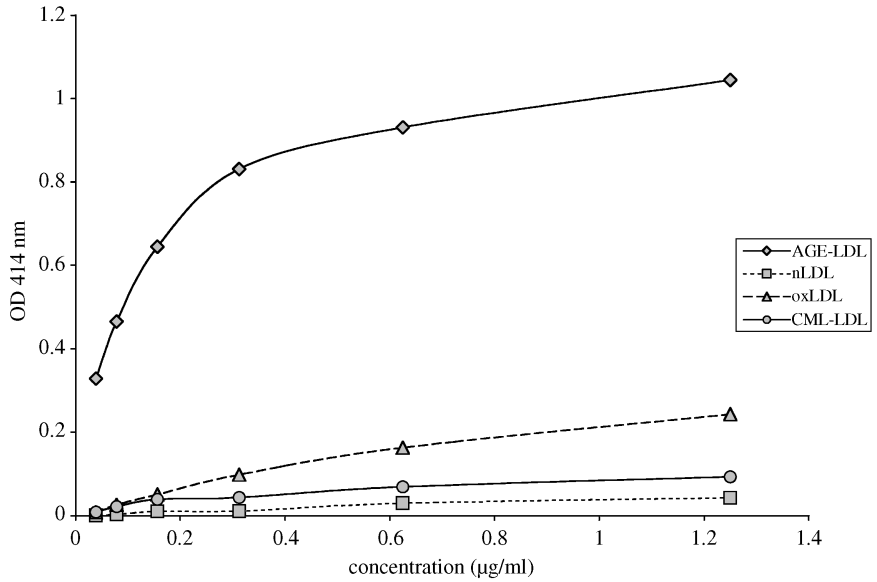


**Fig. 5.** Capture assay with immobilized rabbit AGE-LDL antibody. The plot represents the OD at 414 nm obtained after incubating modified forms of LDL and native LDL (nLDL) preparations at variable concentrations with the immobilized antibody. Peroxidase-labeled anti-human apoB was used to detect the captured LDL.

ies isolated from PEG-precipitated ICs. Of interest is the fact that human OxLDL antibodies are highly specific for OxLDL and MDA-LDL, showing minimal cross-reactivity with MDA-BSA and virtually no reactivity with native LDL. This pattern of reactivity is similar to that reported by other investigators with a human MDA-LDL antibody (18), which reacted both with MDA-LDL and OxLDL and showed a low degree of cross-reactivity with MDA-BSA. On the other hand, AGE-LDL antibodies showed a marked preference for AGE-LDL relative to CML-LDL and cross-reacted with OxLDL. The preferential recognition of AGE-LDL over CML-LDL and the total lack of reactivity with CML-BSA suggests that human AGE-LDL antibodies recognize LDL-specific epitope(s) and that the configuration of the CML epitope(s) may not be identical in AGE-LDL and CML-LDL, perhaps as a consequence of different degrees of modification. In fact, apoB-containing lipoproteins separated as ICs from patient sera contain significantly lower levels of CML per mole of lysine than laboratory-generated AGE-LDL; higher degrees of

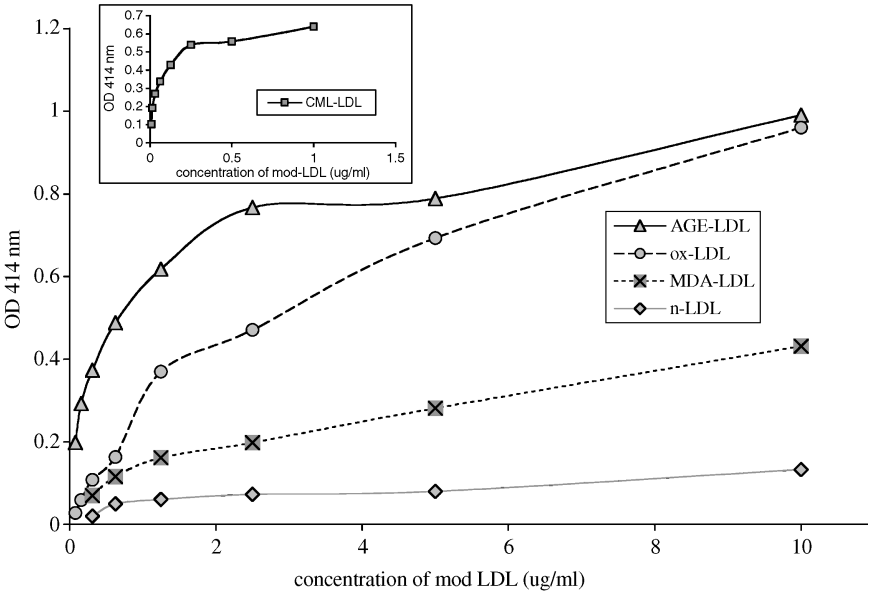


**Fig. 6.** Capture assay with immobilized rabbit CML-LDL antibody. The plots represent the OD at 414 nm obtained after incubating CML-LDL (inset) and other modified forms of LDL and native LDL (nLDL) preparations at variable concentrations with the immobilized antibody. Peroxidase-labeled anti-human apoB was used to detect the captured LDL.

TABLE 4. Results of capture assays of apoB-rich lipoproteins separated after 4% PEG precipitation of LDL-human immune complexes from the supernatant (soluble apoB-rich lipoproteins) and from the precipitates with rabbit antibodies to MDA-LDL, OxLDL, CML-LDL, and AGE-LDL

| Capture Antibody | Soluble apoB-Rich Lipoproteins (n = 12) | apoB-Rich Lipoproteins Isolated from 4% PEG Precipitates (n = 12) | *P* |
|---|---|---|---|
| Rabbit anti-OxLDL | 0.037 ± 0.005 | 0.609 ± 0.052 | <0.0001 |
| Rabbit anti-MDA-LDL | 0.023 ± 0.003 | 0.268 ± 0.038 | <0.0001 |
| Rabbit anti-AGE-LDL | 0.098 ± 0.015 | 0.523 ± 0.052 | <0.0001 |
| Rabbit anti-CML-LDL | 0.068 ± 0.007 | 0.130 ± 0.01 | <0.0001 |

apoB, apolipoprotein B; PEG, polyethylene glycol. Data are expressed as average optical density values at 414 nm ± SEM. The concentration of lipoprotein used in the capture assays was kept constant at 5 μg/ml. Statistical analysis was carried out using the two-tailed paired *t*-test.

CML modification could interfere by charge effects or by steric hindrance with the reactivity of AGE-LDL autoantibodies. The reactivity of human AGE-LDL antibodies with OxLDL seems to be a result of the presence of low concentrations of CML in OxLDL, concentrations very similar to those measured in the apoB-containing lipoproteins coprecipitated with ICs.

The reactivity of rabbit antibodies obtained after immunization with human MDA-LDL and human OxLDL was quite different from that of human OxLDL antibodies. Although rabbit MDA-LDL antibodies reacted equally well with different MDA-modified proteins and cross-reacted with OxLDL, human OxLDL antibodies seem to recognize MDA-lysine epitopes shared by OxLDL and MDA-LDL but not by other MDA-modified proteins.

Rabbit OxLDL antibodies had different patterns of reactivity when tested in competition and capture assays, probably as a consequence of the existence of several populations of antibodies reacting with different epitopes with different affinities. The competition assays, in which the binding of a given antibody to the corresponding antigen is inhibited by the addition of different antigens in relatively high concentrations, favor the detection of low-affinity antibodies. In contrast, capture assays require a higher affinity interaction between antigen and antibody, sufficient to keep the antigen and antibody as a complex through two washing cycles. Thus, low-affinity antibodies to MDA and CML-LDL would explain the extensive cross-reactivity with different types of modified LDL and native LDL. MDA-lysine epitopes are present in MDA-LDL and OxLDL (5), whereas CML-LDL epitopes can be detected in OxLDL, AGE-LDL, and native LDL, albeit over a wide range of concentrations (5, 16).

On the other hand, antibodies of higher affinity to a unique epitope of OxLDL would explain the results observed in the capture assays that indicate that rabbits recognize immunogenic structures of OxLDL that are not shared by MDA-LDL. A possible explanation is that MDA-LDL has two sets of epitopes, one shared with OxLDL that is immunodominant in humans, and one that is shared by different MDA-modified proteins and is immunodominant in rabbits. The higher degree of modification of MDA-LDL (20–25% substitution of lysine residues) may create clusters of MDA lysine that rabbits recognize as an epitope present in other MDA-modified proteins but absent in less modified OxLDL (0.4–0.7% substitution of lysine residues). The epitope recognized by rabbit OxLDL antibodies should also be different from the MDA-related epitope recognized by human OxLDL antibodies, because human antibodies react with both OxLDL and MDA-LDL. A second possibility to be considered is that rabbit OxLDL antibodies recognize epitopes not related to MDA-lysine, such as the one described by Palinski and coworkers (28).

Human AGE-LDL autoantibodies and rabbit AGE-LDL antibodies are protein-specific, because they react primarily with AGE-LDL and cross-react with CML-LDL but do not react with CML-HSA. Immunization of rabbits with human CML-LDL seems to result in the production of more than one antibody population. When tested by competition against immobilized CML-LDL and by capture assay, we detected a high-affinity antibody reactive primarily with CML-LDL but also reacting strongly with CML-BSA, moderately with AGE-LDL, and weakly with OxLDL. The lack of protein specificity of CML antibodies has been reported by other groups (19). When tested against immobilized AGE-LDL, the preferential reactivity with CML-LDL is not obvious and the cross-reactivity with OxLDL is enhanced, suggesting that the assay detects lower affinity antibodies that cross-react more extensively. The cross-reactivity with OxLDL seems to be a consequence of the generation of CML during copper oxidation of LDL, as proven by the isolation of CML-LDL antibodies from an immobilized LDL column.

Rabbit AGE-LDL antibodies are considerably more specific for AGE-LDL than are human antibodies (Table 3), although the capture assay showed definite cross-reactivity with OxLDL. The cross-reactivity with OxLDL could also be explained by the presence of CML in OxLDL, although this is not supported by the lack of reactivity with CML-LDL. Differences in epitope configuration related to the extent of CML modification could be responsible for the different reactivity with CML epitopes in OxLDL and CML-LDL, but it is also possible that rabbit AGE-LDL antibodies may recognize a non-CML epitope also expressed by OxLDL.

The differences in reactivity between rabbit antibodies to different modifications of LDL and human autoantibodies may result from differences between the epitopes generated during spontaneous oxidation in vivo and the epitopes generated by chemical modification of lipoproteins in the laboratory, as discussed above. The possibility that immunogenic epitopes other than MDA may be present in OxLDL and epitopes other than CML may be present in AGE-LDL is strongly suggested by the data generated with rabbit antibodies and needs to be further investigated. But the data generated by capture assays showing that modified lipoproteins isolated from PEG-precipitated ICs were specifically captured with all of the different rabbit modified LDL antibodies tested strongly suggests that there are species-specific differences in the recognition of

different epitopes of modified forms of LDL. Thus, the definition of the immunogenic modifications and epitopes recognized by the human immune system has to be based on the characterization of human autoantibodies rather than on the study of antibodies induced in laboratory animals with in vitro-modified LDL. On the other hand, when developing antibodies for the detection of modified lipoproteins in human samples, laboratory-modified lipoproteins seem to be perfectly adequate, because the epitopes of in vitro-modified LDL recognized by rabbits are also present in modified LDL isolated from human sera.

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