An immunochemical marker of low density lipoprotein oxidation

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Abstract Using monoclonal antibodies against apolipoprotein B (apoB) we studied changes in apoB immunoreactivity during copper ion-mediated oxidation of human low density lipoprotein (LDL). The radioimmunoassay experiments demonstrated the decrease of immunoreactivity of three different epitopes of apoB located in different parts of the protein; at the same time the immunoreactivity of another epitope, previously mapped to the C-terminal 20 amino acids of apoB increased markedly during the first 6 h of LDL oxidation and diminished gradually upon prolonged incubation with copper ions. The fate of LDL during oxidation was also monitored using electrophoretic techniques combined with immunodetection. These experiments showed a rapid fragmentation and disappearance of immunoreactive apoB. They also indicated that the diminishing LDL immunoreactivity detectable during oxidation is associated with apoB fragments still attached to the lipid core. The changes in apoB immunoreactivity during Cu^{2+} treatment of LDL are similar to those observed upon LDL aging. Therefore, it appears that the enhancement of immunoreactivity of the C-terminus of apoB is a general phenomenon associated with various kinds of oxidative modifications of LDL.—**Zawadzki, Z., R. W. Milne, and Y. L. Marcel.** An immunochemical marker of low density lipoprotein oxidation. *J. Lipid Res.* 1989. **30:** 885–891.

Supplementary key words monoclonal antibodies • apolipoprotein B immunoreactivity • oxidative modification of apoB • modified LDL

Several modifications can transform human low density lipoprotein into a form recognized and taken up by macrophages (1–5). The uptake, mediated mainly by the scavenger receptor (4–6), can transform macrophages in vitro into foam cells, identical with those found in atherosclerotic plaques (7, 8). Several lines of evidence indicate that the same mechanism operates in human arteries (8–10) and thus the in vivo modification of LDL may initiate an important atherogenic pathway.

Among the modifications leading to the LDL uptake by macrophages, oxidation is of particular interest. The existing experimental data show that in contrast to some other modifications (e.g., acetylation) oxidation can and most likely does occur in vivo (11–13). The process has been studied extensively. The most commonly used experimental models of LDL oxidation are endothelial cell-mediated modification and incubation with transition metal ions. Both procedures effect the same changes in LDL physicochemical properties [increase in buoyant density and in electrophoretic mobility in agarose (4,5,14,15), phosphatidylcholine hydrolysis (16), loss of cholesteryl esters (17) and polyunsaturated fatty acids (18), and fragmentation of apoB (16, 19)]. Both oxidation procedures also modify the pattern of LDL interactions with cell receptors [recognition by scavenger receptor (4, 5) and loss of recognition by LDL receptor (20)]. Comparatively little is known about the immunochemical properties of oxidized LDL. Steinbrecher et al. (20) reported that immunoreactivity of LDL apolipoprotein B did not change upon oxidation when assessed with polyclonal antiserum. Avogaro et al. (10), using monoclonal antibodies produced in our laboratory, found decreased immunoreactivity in a fraction of LDL isolated from human plasma, with several characteristics of oxidized LDL.

Having at our disposal a large panel of anti-apoB monoclonal antibodies that have been well characterized (21–24), we were particularly interested in finding an immunochemical marker of LDL oxidation, i.e., an apoB epitope whose expression is specifically and significantly altered upon oxidation. We presently report that one of our antibodies appears to detect changes in apoB structure related to oxidation of LDL.

MATERIALS AND METHODS

Isolation of lipoproteins

Blood from normolipidemic humans was collected into EDTA-containing Vacutainers and centrifuged at 2000 *g*

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoB, apolipoprotein B; MAB, monoclonal antibody; RIA, radioimmunoassay; BHT, butylated hydroxytoluene; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylene diaminetetraacetic acid; NCP, nitro- cellulose paper; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

for 15 min to separate plasma. LDL was obtained by sequential ultracentrifugation (1.020 g/ml < d < 1.050 g/ml). Throughout the isolation the plasma contained 10 μM EDTA. Isolated LDL was dialyzed against PBS (10 μM EDTA) and the protein was measured according to the method of Lowry et al. (25). The dialyzed lipoprotein was used in oxidation experiments without further delay.

VLDL (d < 1.006 g/ml) was obtained by density gradient ultracentrifugation of fresh plasma and dialyzed against the same buffer as LDL.

Oxidation of LDL

The procedure of Steinbrecher et al. (20, 26) was used for the copper ion-mediated oxidation of LDL. It consists of exposure of the lipoprotein to 5 μM Cu^{2+} in PBS at 37°C; 50 μM Cu^{2+} was used in some experiments. The process was arrested by addition of 200 μM EDTA and 40 μM butylhydroxytoluene. Oxidized LDL was dialyzed against PBS, 200 μM EDTA, 40 μM BHT. LDL incubated with 200 μM EDTA, 40 μM BHT, and no Cu^{2+} was included as a control. The same protocol was used for Cu^{2+}-treatment of VLDL.

Monoclonal antibodies and antisera[1]

The production and characterization of anti-apoB monoclonal antibodies have been described earlier (21–23). Affinity-purified rabbit anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD).

Solid phase radioimmunoassay of apoB

The assay was based on competition between normal LDL immobilized on polystyrene wells (Removawells, Dynatech Laboratories Inc., Alexandria, VA) and different concentrations of the sample to be tested for each antibody at its predetermined optimal dilution, as described earlier (24). Maximum binding was determined in wells in which no competing soluble antigen was added. The results are expressed as B/B_{max} ratio. Each point is the mean of two measurements (C.V. < 10%).

Polyacrylamide gel electrophoresis

The apoB fragmentation was studied on 1.5-mm-thick 5–15% polyacrylamide gradient slab gels containing SDS (27). The samples were heated for 5 min at 100°C in 50 mM Tris, pH 6.8, 3% SDS, 20% glycerol, 10% 2-mercaptoethanol. The electrophoresis was carried out at 30 mA per gel until the bromophenol blue marker reached the bottom of the gel. The proteins were either stained with Coomassie blue R-250 or transferred to nitrocellulose paper (NCP) (see below).

Nondenaturing 2–16% polyacrylamide gradient gels (Pharmacia, PAA 2/16, Sweden) were also used, according to Gambert (28). After 30 min of preelectrophoresis (4°C, 14 mM Tris, 100 mM glycine, 0.02% NaN_3, pH 8.3, 50 V) the samples were applied and the electrophoresis was carried out at 30 V for 1 h, then at 80 V for 18 h.

The same buffer was used for the electrophoretic transfer of proteins from nondenaturing gels to nitrocellulose paper (0.45 μM pore size, Millipore) according to Towbin, Staehlin, and Gordon (29). The transfer from SDS gels was performed in 20 mM Tris, 150 mM glycine, pH 8.3, 30% methanol (v/v).

The nitrocellulose replicas were used for immunochemical detection of proteins as described earlier (21). Incubations with anti-apoB antibodies were made at 37°C in 10 mM Tris, 150 mM NaCl, 0.01% NaN_3, pH 7.4, containing 1% BSA. ^{125}I-Labeled second antibody (rabbit anti-mouse IgG or goat anti-rabbit serum) was added subsequently and the NCP was autoradiographed on XAR-5 Kodak films with an intensifier screen (Cronex, Dupont).

Agarose gel electrophoresis

Paragon Lipo gels (Beckman) were used according to the instructions of the manufacturer. The transfer of lipoproteins to nitrocellulose was effected by providing contact between the gel and a wet NCP sheet immediately after electrophoresis. After 5 min the transfer was complete as judged by the Sudan black staining of the gel. The immunodetection of proteins on the NCP was performed as described for PAGE.

RESULTS AND DISCUSSION

The changes in immunoreactivity of LDL apoB during incubation with copper ions are presented in **Fig. 1**. A gradual decrease in immunoreactivity with MABs 1D1, 2D8, and 4G3 with increasing incubation time can be seen in panels A–C. The epitopes for these antibodies were previously mapped to the apoB regions of amino acid residues 400–581 (1D1), 1297–1327, and 1407–1479 (2D8), and 2656–3035 (4G3) (23). The decrease was accompanied by a gradual increase in agarose gel electrophoretic mobility of the Cu^{2+}-treated lipoprotein **(Fig. 2)**, a well established characteristic of oxidized LDL (4, 5, 14, 30). In contrast, the reactivity with antibody $B_{sol}7$, whose epitope has been mapped to the C-terminal 20 amino acids of apoB (31), was gradually enhanced during the first 6 h of incubation (Fig. 1D). The effect was partially reversed upon prolonged incubation with Cu^{2+}. The same pattern of immunoreactivity changes was reproduced upon incubation of numerous LDL preparations: 5 μM or

[1]Samples of antibodies 1D1, 2D8, 4G3, and $B_{sol}7$ can be obtained from our laboratory.

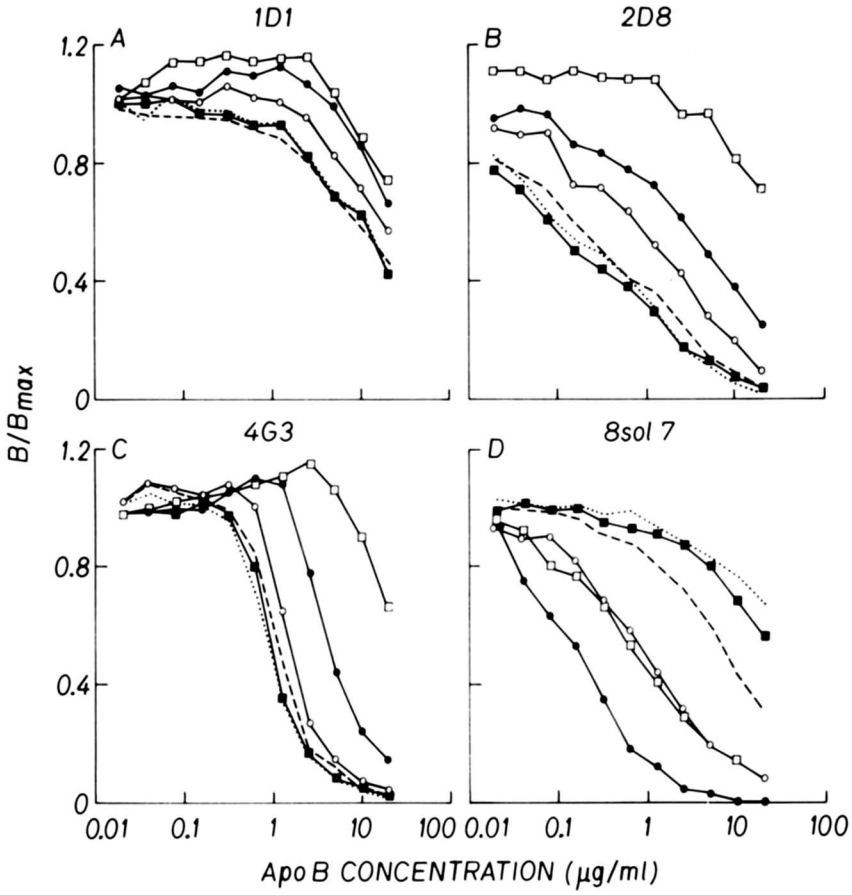

Fig. 1. Competitive RIA studies of changes in apoB immunoreactivity during the CU^{2+}-mediated oxidation of LDL. Four different monoclonal antibodies were used: 1D1 (panel A), 2D8 (panel B), 4G3 (panel C), and $B_{sol}7$ (panel D). Oxidation times studied were: 0 h (.....), 1 h (----), 3 h (-○-○-), 6 h (-●-●-), and 24 h (-□-□-); the solid square curves (-■-■-) represent control 24 h incubations in the presence of EDTA and BHT and without Cu^{2+}.

50 μM Cu^{2+} in the incubation mixture produced the same results.

To determine the specificity of increased $B_{sol}7$ epitope expression in oxidized LDL, the VLDL from two donors was studied in a similar experiment (not illustrated). The RIA using $B_{sol}7$ and 4G3 failed to detect any effect of copper treatment on the expression of either epitope. The immunoreactivity of VLDL with $B_{sol}7$, unlike that with 4G3, was very poor, resembling that of fresh LDL. Assuming that apoB constitutes 30% of VLDL apoprotein, the apoB concentration required for 50% displacement was 27.3 μg/ml with one VLDL preparation. With the other one the displacement in the concentration range studied did not reach 50%. In a typical experiment the value for fresh LDL was 14.0 μg/ml and 0.19 μg/ml for LDL oxidized for 6 h.

The SDS-PAGE followed by immunoblotting technique was used to monitor the fate of apoB during the Cu^{2+}-mediated oxidation of LDL. It has been established (16, 19) that apoB undergoes an extensive fragmentation during LDL oxidation. The rate of disappearance of immunoreactive apoB in our experiments was, neverthelesss, surprising. With some LDL preparations there was virtually no intact 4G3- or $B_{sol}7$- reacting apoB left after 15 min of

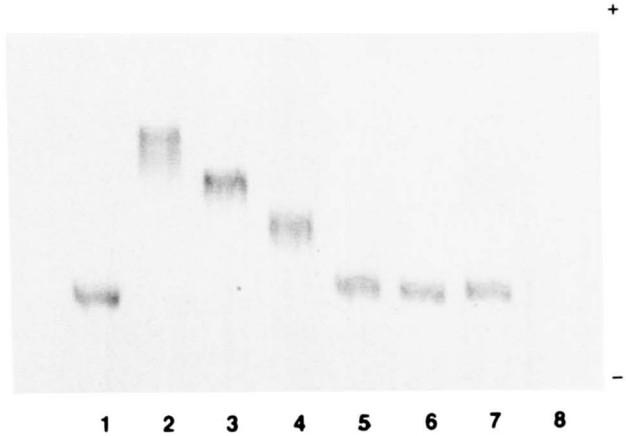

Fig. 2. Agarose gel electrophoresis of LDL incubated with Cu^{2+} for: 0 h (lane 1), 24 h (lane 2), 6 h (lane 3), 3 h (lane 4), 1 h (lane 5), 15 min. (lane 6); lane 7: control 24 h incubation without Cu^{2+} in the presence of EDTA and BHT. Staining was with Sudan black.

Cu^{2+} treatment; lower molecular weight fragments were generated, and with longer oxidation times a complete loss of immunoreactivity in the incubation mixture could be seen **(Fig. 3)**. Immunoblots obtained with $B_{sol}7$ demonstrated the same pattern of immunoreactivity loss (not illustrated). It is noteworthy that the same result was obtained with two antibodies which in the RIA reacted in a different way to the oxidative modification of LDL.

The immunoblots of nondenaturing polyacrylamide gradient (2–16%) gels **(Fig. 4)** confirm the disappearance of immunoreactive apoB from the molecular weight range corresponding to native LDL. It seems that the immunoreactive fragments of apoB generated upon LDL oxidation and dissociated from the lipoprotein particles by SDS treatment remain attached to the lipid core under nondenaturing conditions. The immunoblot in Fig. 4 also reveals the presence of high molecular weight aggregates of LDL in Cu^{2+}-treated lipoprotein. We could detect such aggregates in some, but not all, preparations of oxidized LDL. The formation of aggregates has been described in malondialdehyde- or 4-hydroxynonenal-treated LDL (32, 33) but in those cases the cross-linking of apoB molecules could be documented by the presence of high molecular weight protein in SDS gels. As shown in Fig. 3 we did not observe apoB aggregates under denaturing conditions. It is interesting that the high molecular weight immunoreactive forms of LDL can be found after 6 and 24 h of oxidation with Cu^{2+}, although by the time the LDL did not contain any intact apoB reacting with $B_{sol}7$ or 4G3 (Fig. 3). This suggests that LDL particles are cross-linked via apoB fragments that remain lipoprotein-bound.

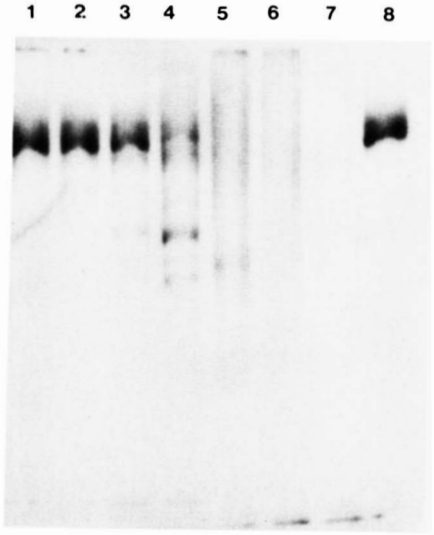

Fig. 3. LDL oxidation products immunodetected by the antibody 4G3 on a blot of 5–15% gradient SDS polyacrylamide gel. Lane 1: control 24 h incubation without Cu^{2+} in the presence of EDTA and BHT; LDL oxidation times were: 0 h (lane 2), 15 min. (lane 3), 1 h (lane 4), 3 h (lane 5), 6 h (lane 6), 24 h (lane 7). Lane 8: untreated LDL after isolation from plasma.

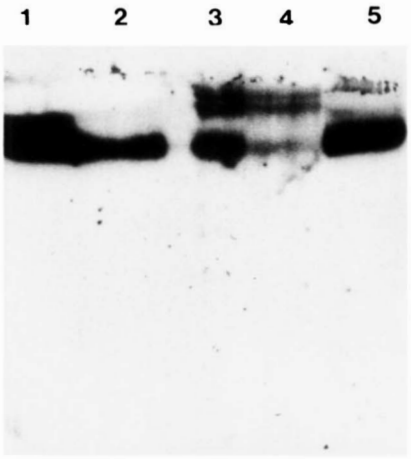

Fig. 4. LDL oxidation products immunodetected by the antibody 4G3 on a blot of 2–16% gradient nondenaturing polyacrylamide gel. LDL was oxidized for: 0 h (lane 1), 1 h (lane 2), 6 h (lane 3), 24 h (lane 4); control 24 h incubation without Cu^{2+} in the presence of EDTA and BHT in lane 5.

The immunoblots of agarose gels of the oxidized LDL **(Fig. 5A–D)** confirm that the particles of Cu^{2+}-modified, faster migrating LDL (and not released apoB fragments) are responsible for whatever immunoreactivity remains in the incubation mixture, as detected by the RIA. The immunoblot shown in Fig. 5D does not indicate the same increase in $B_{sol}7$ epitope expression as could be seen in the RIA. The conditions of the antigen–antibody reaction in these two experiments were different. In the RIA the competing antigen (fresh or oxidized LDL) reacts in solution with $B_{sol}7$ while the immunoblotting technique employs the antigen immobilized on the nitrocellulose paper. As indicated in previous reports from our laboratory (34), a difference of this kind may be the source of conformational changes, resulting in different expression of certain epitopes. The apparent discrepancy between the RIA using $B_{sol}7$ and the immunoblot in Fig. 5D may be another illustration of conformational differences between soluble and solid phase-adsorbed LDL.

The blot presented in Fig. 5E was obtained using a rabbit polyclonal antibody (a gift from Dr. T. Innerarity) against a synthetic peptide, representing the C-terminal 20 amino acids of apoB. (The gel in Fig. 5E was electrophoresed under different conditions than the gels in Fig. 5A–D which resulted in lower mobility of the bands.) It can be seen that this antibody, like $B_{sol}7$ and unlike the three other MABs reacts better with oxidized than with native LDL. Along with the RIA results for $B_{sol}7$ this suggests that despite the very rapid and extensive fragmentation of apoB, resulting in the loss of immunoreactivity of at least three epitopes located in different regions of the molecule, the immunoreactivity of the C-terminus is enhanced, at least during the initial stages of the Cu^{2+} treatment. Presently we can only speculate about the

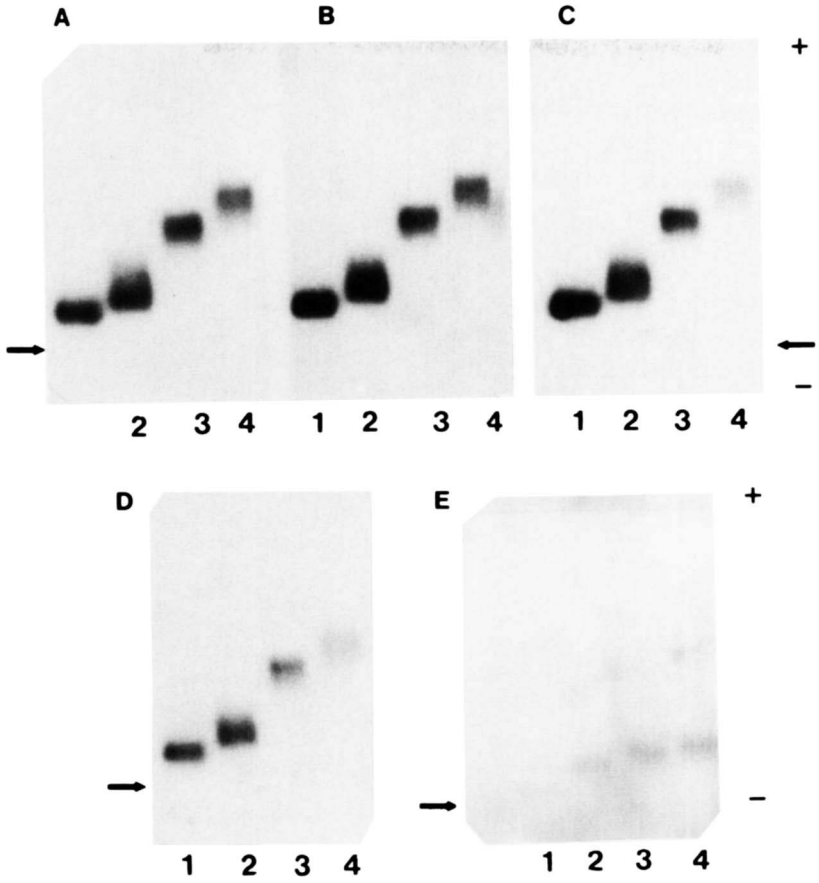

Fig. 5. LDL oxidation products immunodetected by antibodies 1D1 (panel A), 2D8 (panel B), 4G3 (panel C), $B_{sol}7$ (panel D), and anti-C-terminus (see text) (panel E) on blots of agarose gels. LDL oxidation times were: 0 h (lane 1), 1 h (lane 2), 6 h (lane 3), and 24 h (lane 4). Arrows mark the origin line.

possible mechanism of that enhancement. The oxidative modification of LDL apoB appears to consist of at least two distinct processes: the derivatization of some amino acids, most likely lysine (20, 26), by the products of lipid peroxidation, and the oxidative cleavage of the polypeptide chain (35). Both processes can affect the immunoreactivity of apoB epitopes. The changes shown in Fig. 1D suggest that the antibody $B_{sol}7$ detects two phases of oxidative modification of apoB C-teminal region. There are lysines in the epitope region at positions 4513, 4514, and 4526. It is conceivable that the modification of some of them can enhance the immunoreactivity of the epitope $B_{sol}7$. This can be followed by the cleavage, destroying the epitope and reversing the effect of the modification. Another possibility is that the fragmentation of apoB exposes the C-terminal peptide, previously buried in lipids, or changes its conformation in a way that facilitates the reaction with the antibody. Further cleavage (or modification) can destroy the newly exposed epitope. The sequence 4504-4512 is highly hydrophobic (six hydrophobic residues out of nine). Likewise, the C-terminal 5 amino acids include four hydrophobic residues and are probably buried in lipids. Since we previously observed a higher immunoreactivity of $B_{sol}7$ with the peptide 4506–4536 than with 4516–4536 (31) we may hypothesize that Cu^{2+} treatment introduces a first cleavage upstream of residue 4506, generating the immunoreactive epitope for $B_{sol}7$. Subsequent cleavage(s) may occur between the two hydrophobic domains, decreasing the immunoreactivity while leaving the peptide associated with LDL.

The aggregation of LDL during oxidation (Fig. 3) is another possible source of immunoreactivity changes, but it appears that the aggregation alone cannot account for all our observations. In our experiments the immunoreactivity changes were not always accompanied by a detectable level of LDL aggregation. Also, the increase in $B_{sol}7$ epitope expression is not readily explained by LDL aggregation. It is conceivable that both these phenomena result from the same type of modification of lipoprotein structure (e.g., loss of some surface components of LDL).

Our interest in the interactions of the oxidized LDL with antibody $B_{sol}7$ resulted from an earlier observation (Ruys, D. and Marcel, Y. L., unpublished data) confirmed in this study and in previous ones (31) that LDL prepara-

tions stored for several months at 4°C reacted much better with that antibody than did native LDL. The oxidative modification of apoB was one of the most likely explanations for that change in immunoreactivity. The present study confirms that the LDL oxidation affects the immunochemical expression of apoB. It should be borne in mind that the antibody $B_{sol}7$, unlike other antibodies used in the RIAs reported above, was obtained after immunization of the mice with soluble apoB and not with intact LDL (34). It is possible that the protein may have undergone a certain degree of oxidation during the delipidation and solubilization procedure. That could explain the preferential reactivity of $B_{sol}7$ with oxidized LDL.

The enhanced immunoreactivity of the epitope for $B_{sol}7$ becomes a candidate marker for the apoB modification accompanying LDL oxidation, in vitro as well as in vivo.

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