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Low density lipoprotein undergoes oxidative modification *in vivo*

(atherosclerosis/autoantibodies to oxidized low density lipoprotein/malondialdehyde/4-hydroxynonenal)

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Contributed by Daniel Steinberg, November 17, 1988

ABSTRACT **It has been proposed that low density lipoprotein (LDL) must undergo oxidative modification before it can give rise to foam cells, the key component of the fatty streak lesion of atherosclerosis. Oxidation of LDL probably generates a broad spectrum of conjugates between fragments of oxidized fatty acids and apolipoprotein B. We now present three mutually supportive lines of evidence for oxidation of LDL *in vivo*: (*i*) Antibodies against oxidized LDL, malondialdehyde-lysine, or 4-hydroxynonenal-lysine recognize materials in the atherosclerotic lesions of LDL receptor-deficient rabbits; (*ii*) LDL gently extracted from lesions of these rabbits is recognized by an antiserum against malondialdehyde-conjugated LDL; (*iii*) autoantibodies against malondialdehyde-LDL (titers from 512 to >4096) can be demonstrated in rabbit and human sera.**

A growing body of evidence suggests that oxidative modification of low density lipoprotein (LDL) enhances its atherogenicity (for review see ref. 1). Monocyte-derived macrophages, the precursor of most foam cells in early atherosclerotic lesions, cannot take up native LDL rapidly enough to cause lipid loading (2). Oxidative modification converts LDL to a form recognized by the macrophage acetyl-LDL receptor (1, 2) and possibly by other receptors as well (3). This is true whether the oxidation is effected by incubation under appropriate conditions with cultured cells or by autooxidation catalyzed by Cu^{2+} ions in the absence of cells. Oxidative modification of LDL is accompanied by extensive degradation of its polyunsaturated fatty acids, generating a complex array of shorter chain-length fragments (4). During the oxidation, some of these fragments become covalently linked to apolipoprotein B (5), and much of this conjugation involves the ϵ-amino groups of lysine residues. This chemically modified form of apolipoprotein is recognized by the acetyl-LDL receptor (6). Thus we can generate models for oxidized LDL by conjugating the apolipoprotein with single compounds generated during oxidation. Fogelman *et al.* (7) demonstrated that malondialdehyde (MDA)-conjugated LDL is so recognized. If oxidized LDL contains lysine residues conjugated with a variety of fatty acid fragments of different chain lengths, it should react with antibodies against a variety of such lysine derivatives. We previously showed that immunization of animals with autologous LDL modified by conjugation of lysine groups with glucose yields antisera directed specifically against glucitollysine (8) and that antibodies generated by injection of carbamoylated autologous LDL generates antisera that recognize the carbamoyllysine—not only in LDL but in other conjugated proteins as well (9). In other words, the specificity of these antisera is for very narrowly defined "X"-lysine adducts.

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The present studies, which use immunochemical methods, offer three lines of evidence that oxidation of LDL occurs *in vivo*.

METHODS

Lipoprotein Isolation and Modification. LDL (1.019–1.063 g/ml) was prepared from pooled plasma of healthy humans, male guinea pigs, mice, and New Zealand White (NZW) rabbits, using EDTA (1 mg/ml), butylated hydroxytoluene (BHT) (10 μM), and a combination of protease inhibitors (10). MDA-LDL was prepared by incubating LDL with freshly made 0.5 M MDA, generated from malonaldehyde bis(dimethylacetal) by acid hydrolysis at a constant molar ratio of 100 μl/mg of LDL. The degree of MDA modification determined by the thiobarbituric acid method averaged 77% lysine residues. Other proteins were conjugated with MDA similarly. Cu^{2+}-oxidized LDL was prepared as described (11). 4-Hydroxynonenal (4-HNE)-LDL was prepared by incubating LDL with 4-HNE by a modification of the method of Esterbauer *et al.* (12). 4-HNE was provided by Hermann Esterbauer (Graz, Austria).

Mouse LDL was oxidized by exposure to 10 μM Cu^{2+} for 18 hr as described (11), delipidated with methanol and chloroform, and the resulting protein fragments were resolubilized in 55 mM octyl glucoside as described (6).

Immunization Protocol and Antibody Characterization. Guinea pigs were immunized with guinea pig MDA-LDL or guinea pig 4-HNE-LDL as described (9). Solid-phase RIA techniques were used to determine titers and specificity of antibodies to MDA-LDL and to 4-HNE-LDL as described (9) except that the antigen, modified LDL, was plated at 4°C for 4 hr to prevent oxidation. Guinea pig IgG was detected by a goat anti-guinea pig IgG (Cooper Biomedical) (9). The delipidated and solubilized fragments of oxidized murine LDL protein were used to immunize BALB/c mice, and monoclonal antibodies (mAbs) were produced as described (13). In brief, primary screening of the hybridoma supernatants was done against the immunizing agent, intact Cu^{2+}-modified human LDL, and native human LDL. Clones reacting with the oxidized forms were selected. A detailed characterization of this antibody and of the antiserum against 4-HNE-lysine will be presented elsewhere.

Autoantibodies to rabbit MDA-LDL in rabbit sera were titered as described above, except that goat anti-rabbit IgG (Cooper Biomedical) was used as the second antibody. Autoantibodies in human sera were determined similarly, using human albumin (Sigma) instead of bovine serum albumin as the blocking agent and goat anti-human IgG (Cooper Biochemical) as the second antibody. Because no preimmune

Abbreviations: LDL, low density lipoprotein; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; mAb, monoclonal antibody; WHHL, Watanabe heritable hyperlipemic; NZW, New Zealand White; BHT, butylated hydroxytoluene.

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sera were available, titers were defined as the reciprocal of the greatest serum dilution that showed binding three times that observed in wells containing no antigen. The specificity of the antibodies was tested by competition RIA (9, 14).

Tissue Preparation for Lipoprotein Extraction and Immunostaining. Male Watanabe heritable hyperlipemic (WHHL) rabbits, from 4 mo to 4-yr in age, were given ketamine/xylazine i.m. (35 mg/kg and 5 mg/kg, respectively). The aortic tree was perfusion-fixed with 2 liters of formal/sucrose (4% paraformaldehyde/5% sucrose, pH 7.4) containing 50 μM BHT and 1 mM EDTA. Tissue segments for immunostaining were rinsed in 0.1 M sodium phosphate buffer, pH 7.4/4% sucrose/0.15 mM $CaCl_2$/50 μM BHT/1 mM EDTA overnight at 4°C to remove any remaining fixative, dehydrated through a graded series of ethanol concentrations, and embedded in paraffin. In those animals used for both immunostaining and extraction of intimal LDL, the animals were perfused only with buffer containing BHT and EDTA, and the segments for immunostaining were immersion-fixed overnight.

Soluble intimal lipoproteins were extracted from minced atherosclerotic areas, using a described procedure (15) but with the addition of 10 μM probucol as an antioxidant to the extraction buffer. LDL was isolated from this extract by ultracentrifugation as described (15), and plasma LDL was subjected to the same extraction procedures.

Immunocytochemistry. Serial 5-μm-thick sections were cut from the paraffin-embedded aortae, rehydrated, and immunostained using an avidin-biotin-alkaline phosphatase system (Vector Laboratories). Alkaline phosphatase was selected instead of horseradish peroxidase to avoid the need to preblock endogenous peroxidase by exposure to hydrogen peroxide. Adjacent sections were immunostained with antiserum Mal-2 (anti-MDA-lysine), an antiserum to 4-HNE, mAB OLF4-3C10 (anti-oxidized apolipoprotein B fragments), as well as with the apolipoprotein B-specific mAb MB-47 (14). Control studies were done with nonspecific antibodies and with antisera preincubated with plated antigens to absorb specific antibodies. For example, MAL-2 was preincubated successively in 60-mm dishes coated with MDA-LDL (and postcoat) until no titer remained against MDA-LDL. Sections were also stained with RAM-11, which specifically recognizes rabbit macrophages (16) (gift of A. M. Gown, University of Washington).

Western (Immunologic) Blots. Ten micrograms of LDL, isolated from the atherosclerotic lesions of the same aortae used for immunostaining was electrophoresed in a nonreducing 4–12% $NaDodSO_4$/polyacrylamide gel. The proteins were then transblotted to nitrocellulose membranes and incubated with MAL-2, MB-47, or other antisera. Autoradiographs were obtained after incubation with ^{125}I-labeled goat anti-guinea pig IgG (for MAL-2) or goat anti-mouse IgG (for MB-47) (17).

RESULTS

To develop an antiserum that would selectively and specifically recognize MDA-lysine adducts, but not epitopes of native LDL, we immunized guinea pigs with guinea pig MDA-LDL. Antisera with titers $>10^5$ were generated after a primary and two booster injections (n = 4). The specificities of the antisera were tested using solid-phase competitive RIAs, and results with one antiserum (MAL-2) are shown in Fig. 1. Human MDA-LDL was a highly effective competitor. Human MDA-albumin and human MDA-hemoglobin also competed but did so less effectively. Even simple MDA-lysine adducts—MDA-polylysine and MDA-ε-aminocaproic acid—could compete, although to a much more limited extent. Native human LDL was not competitive at all. Thus, this monospecific antiserum is specifically directed to the MDA-lysine epitope, recognizing it on any of a variety of different proteins. An antibody against 4-HNE-LDL was also generated in guinea pigs by similar methods. It, too, specifically recognized the 4-HNE-lysine epitopes present on a variety of different proteins. Finally, a mouse mAb was generated using the delipidated protein fragments of Cu^{2+}-oxidized mouse LDL as antigen. This antibody specifically recognizes Cu^{2+}-modified rabbit and human LDL but not native LDL. A full characterization of the latter two antibodies will be presented elsewhere.

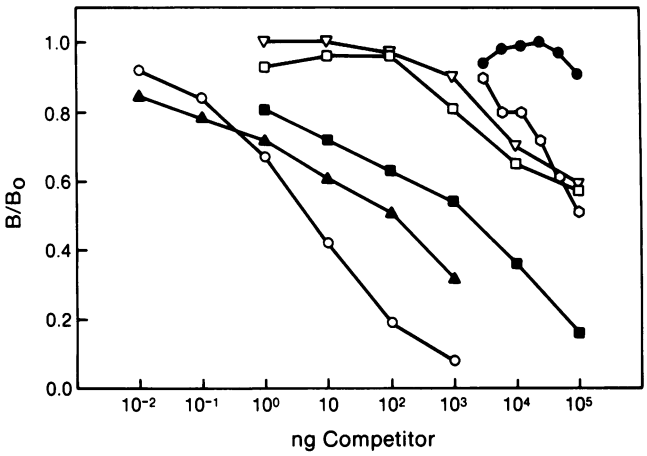

FIG. 1. Solid-phase RIA of MAL-2 with various competitors. Guinea pig MDA-LDL (1 μg/ml) was the plated antigen and a 1:10,000 dilution of MAL-2 was added with and without competitor. Results are expressed as B/B_o, where B is the amount of MAL-2 bound in the presence and B_o that bound without competitor. ○, Human MDA-LDL; ▲, human MDA-albumin; ■, human MDA-hemoglobin; □, MDA-*t*-butoxycarbonyl (tBOC)-lysine; ▽, MDA-ε-aminocaproic acid; ○, MDA-polylysine; and ●, human LDL.

Using the three antibodies described above, we observed considerable immunoreactivity in atherosclerotic lesions from the aorta of a 2-year-old WHHL rabbit. In the representative sections shown in Fig. 2, the immunostaining is largely confined to the lipid-rich shoulder region of an advanced lesion in the aorta. Most importantly, the pattern of immunostaining was similar for all three antibodies. An adjacent section, stained with the macrophage-specific mAb RAM-11 showed that the area stained by these antibodies was rich in macrophages (Fig. 2*E*). Higher magnification of one area of the macrophage-rich lesion shows that the immunostaining with MAL-2 was predominantly intracellular (Fig. 2*F*). Other sections, however, demonstrated extracellular staining as well. When MAL-2 had been preincubated with MDA-LDL to absorb MDA-lysine-specific antibodies, there was no staining (Fig. 2*D*). Similar controls were obtained with preabsorbtion of the other antibodies and with nonimmune sera (data not shown). As an additional control, similar studies were made of aortic sections of nonlesioned areas obtained from young WHHL rabbits. In these sections no immunostaining was observed.

mAb MB-47, which is specific for the receptor-binding region of apolipoprotein B (14), gave heavy immunostaining in the macrophage-rich region of the lesion in Fig. 2 (data not shown); however, while some areas showed a clear coincidence of staining with MAL-2 and mAb MB-47, there was dissociation in other areas. This was particularly true with respect to the intracellular staining of macrophages, which was prominent with MAL-2, OLF4-3C10, and 4-HNE, whereas mAb MB-47 showed little or no intracellular immunostaining.

These immunohistologic data document that MDA-lysine residues are present in arteriosclerotic lesions, in agreement with the findings of Haberland *et al.* (18). However, because the antibodies are specific for MDA- and 4-HNE-lysine residues and relatively independent of which protein they occur in, these results do not necessarily indicate the presence of modified LDL itself.

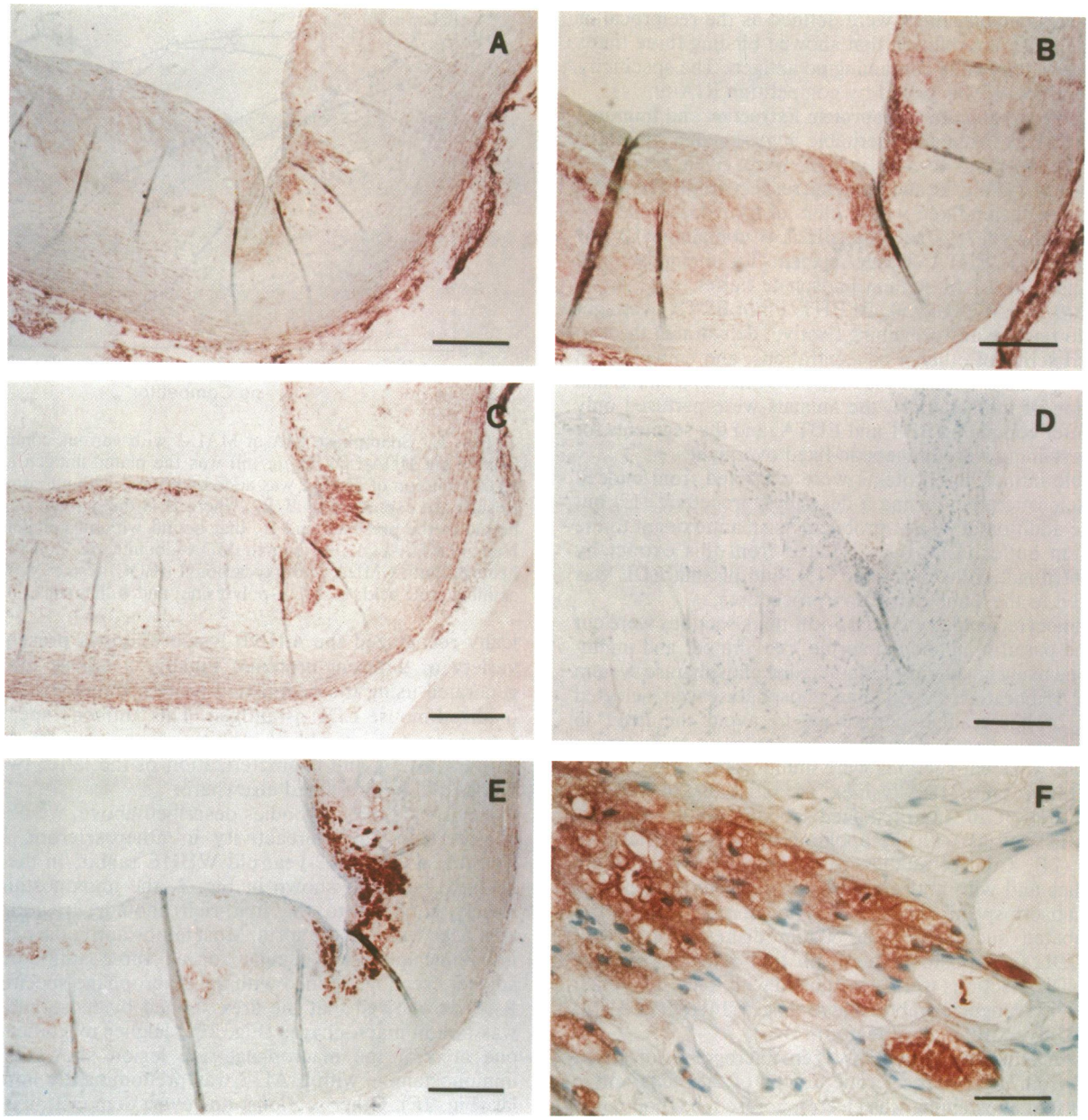

FIG. 2. Alkaline phosphatase immunostaining of an advanced atherosclerotic lesion from the aortic arch of a 2-yr-old WHHL rabbit. Red stain indicates the presence of the primary antibody; the nuclei are counterstained with methyl green. (*A*) Section stained with MAL-2 (1:1000 dilution) shows the localization of MDA-lysine epitopes within the lesion. (*B*) Adjacent section stained with an antiserum specific to 4-HNE (dilution 1:3000). (*C*) Section stained with OLF4-3C10, a mAb against Cu^{2+}-oxidized LDL (dilution 1:1000). (*D*) Control section, stained with a 1:1000 dilution of MAL-2 preincubated with human MDA-LDL to absorb the MDA-lysine-specific antibodies. (*E*) Adjacent section stained with a 1:3000 dilution of the macrophage-specific mAb RAM-11. (*F*) Higher magnification of a macrophage-rich area of the lesion, stained with MAL-2, shows the predominantly intracellular localization of the immunostain. (Bar = 100 μm in *A–E*; bar = 10 μm in *F*.)

To determine whether or not oxidatively modified LDL *per se* was present, LDL was gently eluted from aortic sections using techniques developed by Ylä-Herttuala *et al.* (15). Intimal LDL (ρ = 1.020–1.072 g/ml) was isolated from the aortic extracts by ultracentrifugation and subjected to NaDodSO$_4$/PAGE. Coomassie blue staining documented the presence of intact apolipoprotein B and of fragments resembling those seen after oxidative modification *in vitro* (6). That these lower molecular weight fragments were, indeed, derived from apolipoprotein B was confirmed by Western blot techniques, using a panel of apolipoprotein-B-specific mAbs (10). For example, both the intact apolipoprotein B-100 and a number of lower molecular weight fragments reacted with mAb MB-47 (Fig. 3, lane 1, *Right*). Antiserum MAL-2 reacted with intact apolipoprotein B and also with a lower molecular weight band, also staining with MB-47 (lane 1, *Left*), documenting that *some* apolipoprotein B fragments contained MDA-lysine adducts. Note that the plasma LDL from the same WHHL rabbit, isolated under identical conditions to the intimal LDL, showed no MAL-2-reactive proteins (lane 2). Antisera specific for 4-HNE lysine also bind to apolipoprotein B fragments in LDL obtained from the aortic wall (data not shown). Fig. 3 (lane 3) further shows that Cu^{2+}-catalyzed oxidation of LDL also generates material staining with MAL-2. In other words, oxidation of LDL even without any added MDA generates MDA-lysine adducts, presumably the result of the peroxidation of polyunsaturated fatty acids.

Previous studies in this laboratory have shown that relatively minor modifications of LDL render it highly immuno-

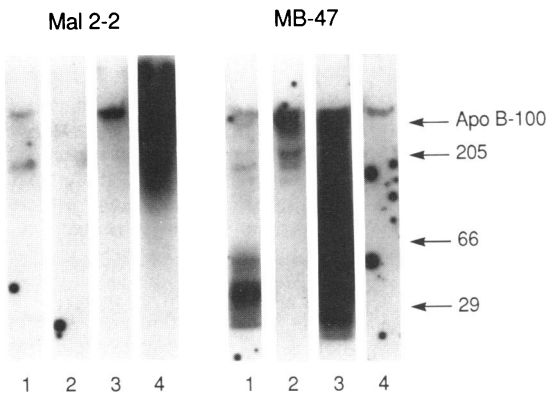

FIG. 3. Western blot of intimal LDL, demonstrating presence of MDA-lysine epitopes in apolipoprotein B. Ten micrograms of LDL protein was applied to each lane of a 4–12% $NaDodSO_4$/PAGE, and then Western blots were performed from this gel using MAL-2 (1:200 dilution, *Left*) and MB-47 (1:1000 dilution, *Right*). Lanes: 1, LDL isolated from atherosclerotic intima of the aorta shown in Fig. 2; 2, LDL isolated from plasma of same rabbit; 3, human LDL oxidized in the presence of 10 μM Cu^{2+} ions for 18 hr; 4, MDA-modified human LDL. Numbers on the right are $M_r \times 10^{-3}$.

genic even in homologous species, and autoantibodies to glucosylated LDL are found in human serum (19). For this reason and in view of the preliminary report by Parums and Mitchinson (20) of human autoantibodies against ceroid pigment, we tested sera of rabbits and patients for such autoantibodies. Surprisingly, every serum tested showed some titer of autoantibody against homologous MDA-LDL. Fig. 4*A* shows representative antibody-binding curves for four control subjects and for four patients with coronary artery disease. Similar binding curves have been determined in 16 additional subjects. All showed titers ranging from 512 to >4096. The titers in patients with coronary artery disease were not higher than those in the control subjects. Sera from WHHL rabbits and from NZW rabbits also showed significant titers of autoantibodies against MDA-LDL. Even the guinea pigs used to generate the antisera against MDA-LDL had preexisting antibodies specific for MDA-lysine (data not shown).

In view of the surprising ubiquity of these autoantibodies, we performed solid-phase competitive RIAs on selected sera from individual rabbits and individual subjects as the source of antibodies. Also, to ensure that the binding proteins were indeed immunoglobulins, the immunoglobulin fraction from one human subject was prepared using a protein A affinity column and used as a source of antibody for the competitive RIA shown in Fig. 5*A*. Fig. 5*B* shows parallel competition studies using whole serum from a NZW rabbit as the source of primary antibody. In both cases, a variety of MDA-modified proteins served as effective competitors, and the relative effectiveness was analogous to that seen with antisera induced against MDA-LDL in guinea pigs (see Fig. 1). Competition studies using whole human serum yielded comparable results (data not shown). The results shown in Fig. 5 are representative of those with a number of rabbit and human samples tested. In each case a somewhat different degree of competition was seen but, in general, all these antibodies recognized primarily the MDA-lysine residues.

DISCUSSION

During the process of lipid peroxidation, a variety of highly reactive aldehyde products are generated, which, in turn, can form covalent bonds with protein, principally lysine residues (1). MDA and 4-HNE are two of these products and readily react with lysine residues. In this report we present immunocytochemical evidence for colocalization within WHHL aortic lesions of MDA-lysine and 4-HNE-lysine epitopes as well as epitopes of oxidized LDL apolipoprotein B. These data are compatible with the presence of oxidized LDL in the atherosclerotic lesion. However, in view of the cross-reactivity of the antibodies against MDA-LDL with other MDA proteins (Fig. 1) and of 4-HNE-LDL with other 4-HNE-proteins, the immunostaining would not necessarily differentiate between conjugates present in apolipoprotein B (or its fragments), and conjugates in other proteins—including membrane proteins.

The demonstration that LDL gently extracted from aortic lesions contains apolipoprotein B-100 and apolipoprotein B fragments reactive with antibodies against MDA-lysine (Fig. 3) and with antibodies against 4-HNE-lysine (data not shown) provides direct evidence that oxidized *LDL itself* is present in the aortic wall. This is also suggested by the observation that immunostaining was seen *only* in lesions and not in unaffected areas of the same aorta. Furthermore, in early fatty streak lesions we find extensive immunostaining in the extracellular

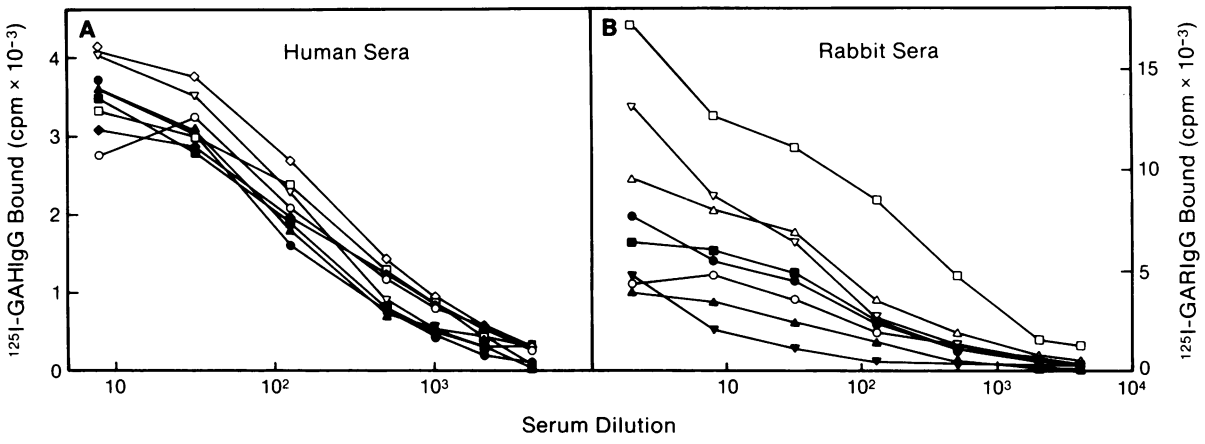

FIG. 4. Representative antibody-binding curves of human sera (*A*) and rabbit sera (*B*) to homologous MDA-LDL. (*A*) Human MDA-LDL was plated at 5 μg/ml, and antibody-binding curves were determined for controls (open symbols) and patients with coronary artery disease (closed symbols). The amount of specific antibody bound, detected with ^{125}I-labeled goat anti-human IgG (GAHIgG), was determined by subtracting the nonspecific binding to human serum albumin. All 12 control sera screened and 10 of 12 patients' sera had titers >4096; the remaining two patients had a titer of 2048. (*B*) Plates were coated with rabbit MDA-LDL (5 μg/ml), and antibody-binding curves were determined for WHHL and NZW rabbits. Representative specific binding curves for four NZW (open symbols) and four WHHL (closed symbols) are shown. Antibody bound was determined by use of ^{125}I-labeled goat anti-rabbit IgG (GARIgG). Of 12 rabbits screened, one WHHL and five NZW rabbits had titers >4096, two WHHL and one NZW had a titer of 2048, and three WHHL had titers of 512. The titers of the rabbit antisera against human MDA-LDL were identical (data not shown).

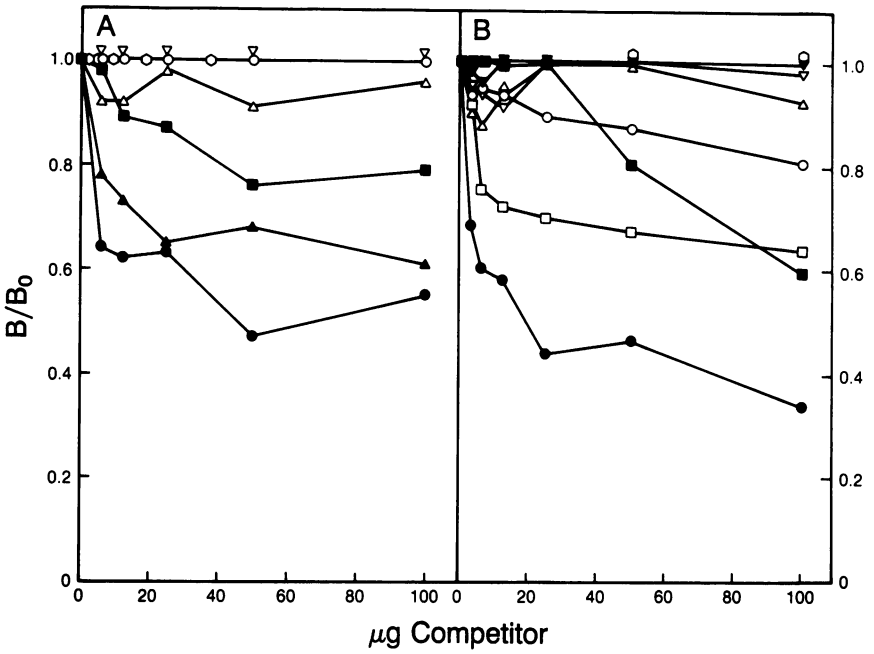

FIG. 5. Specificity of binding to MDA-LDL of human IgG (*A*) and a rabbit serum (*B*). (*A*) Microtiter wells were coated with human MDA-LDL at 1 μg/ml and incubated with a 1:32 dilution of protein A-purified IgG isolated from 50 ml of serum of control subject J.W. with and without the indicated concentrations of competitors. Data are expressed as indicated in the legend to Fig. 1. Competition studies performed with whole serum of J.W. gave similar results. ●, Human MDA-LDL; ▲, human MDA-hemoglobin; ■, human MDA-transferrin; △, MDA-polylysine; ○, MDA-albumin; ▽, human LDL; ○, human Cu^{2+}-oxidized LDL. (*B*) Microtiter wells were coated with rabbit MDA-LDL (1 μg/ml) and incubated with a 1:128 dilution of serum from a NZW rabbit in the absence and presence of competitors. ●, Human MDA-LDL; □, rabbit MDA-LDL; ■, rabbit MDA-transferrin; ○, human LDL, stored for 8 weeks; △, rabbit LDL; ▽, human MDA-transferrin; ▼, MDA-polylysine; ○, acetyl-LDL.

spaces with both mAb MB-47, indicative of apolipoprotein B, and antibodies against oxidized forms of LDL.

MDA-LDL in the aortic wall could be the immunogen giving rise to the autoantibodies reported here. The endogenous immunogens need not be exclusively modified forms of LDL; other conjugates could, in principle, give rise to these antibodies, which would then recognize modified LDL along with the parent immunogenic proteins. However, previous studies from this laboratory have shown that modified LDL is much more effective as an immunogen than analogously modified albumin, transferrin, or other proteins tested (8, 9), making modified LDL a more likely candidate for the immunogen.

The three lines of evidence presented here—immunostaining of aortic lesions with antibodies against oxidized forms of LDL, isolation of oxidized LDL directly from aortic lesions, and demonstration of autoantibodies against MDA-LDL suggests that LDL undergoes oxidative modification *in vivo*, that it accumulates in aortic lesions, and that it may be a source of autoantibody production even in normal animals and normal human subjects.

These findings in themselves do not establish a pathogenetic role for oxidized LDL but only its occurrence *in vivo*. However, the fact that antioxidant therapy slows the progress of early atherosclerotic lesions (21) has provided evidence that oxidized LDL *does* play a pathogenetic role by one of several mechanisms previously discussed (1). The finding that autoantibodies against MDA-LDL are widely prevalent suggests still another mechanism by which oxidized LDL may be pathogenetic. Immune complexes can be taken up avidly into the macrophage via the Fc receptor and it may require only moderate degrees of lysine conjugation for this to occur. Studies by Haberland *et al.* (22) show that 20% or more of the lysine residues on apolipoprotein B must be conjugated with MDA to generate the site recognized by the acetyl-LDL receptor. However, lesser degrees of lysine modification may suffice to generate immune complexes and accelerate macrophage uptake by this mechanism (23). A number of other adducts of LDL with fragments of oxidized fatty acids must occur, such as 4-HNE-lysine, and our preliminary data suggest that autoantibodies to these exist as well. Possibly some of the high concentrations of immunoglobulin present in the artery wall (24, 25) may represent such immune complexes.

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