# Cloning of Monoclonal Autoantibodies to Epitopes of Oxidized Lipoproteins from Apolipoprotein E-deficient Mice

## Demonstration of Epitopes of Oxidized Low Density Lipoprotein in Human Plasma

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## Abstract

**Many reactive products may be formed when LDL undergoes lipid peroxidation, which in turn can react with lipids, apoproteins, and proteins, generating immunogenic neoepitopes. Autoantibodies recognizing model epitopes of oxidized low density lipoprotein, such as malondialdehyde-lysine, occur in plasma and in atherosclerotic lesions of humans and animals. Because apo E-deficient mice develop particularly high titers of such autoantibodies, we used their spleens to clone 13 monoclonal antibodies to various epitopes of oxidized LDL ("E0 antibodies"). Binding and competitive RIAs demonstrated significant differences in fine specificity even between E0 antibodies initially selected for binding to the same screening antigen. For example, some E0 antibodies selected for binding to malondialdehyde-LDL also recognized copper oxidized LDL, acrolein-LDL, or LDL modified by arachidonic or linoleic acid oxidation products. Circulating IgG and IgM autoantibodies binding to copper-oxidized LDL, 4-hydroxynonenal-LDL, acrolein-LDL, and LDL modified with arachidonic or linoleic acid oxidation products were found in apo E–deficient mice, suggesting that the respective antigens are formed in vivo. Epitopes recognized by some of the E0 monoclonal antibodies were also found on human circulating LDL. Each of the E0 monoclonal antibodies immunostained rabbit and human atherosclerotic lesions, and some of them yielded distinct staining patterns in advanced lesions. Together, this suggests that the natural monoclonal antibodies recognize different epitopes of complex structures formed during oxidation of lipoproteins, or epitopes formed independently at different lesion sites. Our data demonstrate that a profound immunological response to a large number of different epitopes of oxidized lipoproteins occurs in vivo. The availability of "natural" monoclonal autoantibodies should facilitate the identification of specific epitopes inducing this response.**

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## Introduction

Substantial evidence indicates that lipoproteins modified by oxidation or nonenzymatic glycation can contribute to atherogenesis (reviewed in references 1–4). During oxidative modification of LDL, highly reactive lipid peroxidation products, such as malondialdehyde (MDA),[1] form adducts with free amino groups of lysines and other amino acids of apo B. Modified apo B is highly immunogenic (5–7), and circulating autoantibodies to epitopes of oxidized LDL (OxLDL), such as MDA-lysine, have been demonstrated in the plasma of humans, rabbits, and mice (6, 8–10). A number of studies have suggested that higher titers of these autoantibodies are found in patients with increased carotid atherosclerosis, coronary artery disease, diabetes, peripheral vascular disease, hypertension, and preeclampsia (11–20). The autoantibodies recognize epitopes of OxLDL in atherosclerotic lesions (10, 21), and are present within lesions, in part as immune complexes with OxLDL (22). A prospective study recently demonstrated that the development of autoantibody titers correlates with the extent of atherogenesis in cholesterol-fed LDL receptor–deficient mice (23). This suggests that OxLDL accumulating in lesions may induce autoantibody formation, although it is also possible that the rise in titers reflects enhanced lipid peroxidation in general, particularly in the setting of hypercholesterolemia, which may cause a more generalized enhancement of lipid peroxidation (24, 25). Finally, we have recently shown that induction of very high titers of antibodies by hyperimmunization of LDL receptor–deficient rabbits with homologous MDA-LDL significantly reduced progression of atherosclerosis (26), suggesting that the humoral immune response to OxLDL may actually modulate the atherogenic process.

A large number of reactive lipid peroxidation products are potentially generated during the oxidation of LDL (27–29), and modification of lipids as well as apoproteins undoubtedly occurs in vivo. For example, highly reactive aldehydes can be generated as breakdown products of polyunsaturated fatty acids. These aldehydes can form Schiff base type adducts with lysines of apo B, as well as more complicated structures. Modi-

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1. *Abbreviations used in this paper:* 4-HNE-LDL, 4-hydroxynonenal-modified LDL; AOP (or LOP)-LDL, LDL modified with arachidonic (or linoleic) acid oxidation products; $Cu^{2+}$-LDL, copper-oxidized LDL; GAM-IgG, goat anti–mouse IgG; MDA, malondialdehyde; NR, nonreduced; OxLDL, oxidized LDL; R, reduced.

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fied phospholipids are also generated (30–32). Many of these reaction products may also be immunogenic. To demonstrate the presence of OxLDL in vivo, a number of antisera and monoclonal antibodies have previously been generated against model epitopes of OxLDL, such as MDA-lysine and 4-hydroxynonenal (4-HNE)-lysine, and against unknown epitopes present in copper-oxidized LDL ($Cu^{2+}$-LDL) (6, 7, 33–37). In addition, antibodies to epitopes of hypochlorite-modified LDL have recently been generated (38). Western blot analysis of extracted lipoprotein fractions and immunocytochemistry of atherosclerotic lesions with these antibodies established the occurrence of some of these epitopes or immunologically similar structures in lesions (6, 7, 10, 21, 23, 33–37). Enzymes that may catalyze lipoprotein oxidation, such as myeloperoxidase, have also been demonstrated in lesions (39). However, with the exception of MDA and 4-HNE, the chemical nature of epitopes formed during the oxidation of lipoproteins in vivo is unknown. Even the exact epitope recognized by the monoclonal antibody MDA2 (which binds to MDA-lysine) is not clear because MDA may form monomers, dimers, and complex heterocyclic structures (28).

Similarly, screening for autoantibodies has been carried out with only a small number of antigens. In theory, the availability of autoantibodies makes it possible to quantitate, isolate and characterize epitopes formed in vivo. To date, this approach has been impeded by the relatively low titer of autoantibodies in humans and some animal models of atherosclerosis (6). However, murine models are now available that develop extensive hypercholesterolemia and atherosclerosis in the entire aorta (10, 40–46). We recently observed that autoantibody titers to MDA-lysine were very high in one such model, apo E–deficient mice, compared to C57BL/6 mice (10). This suggested that apo E–deficient mice would also have high titers of autoantibodies to other epitopes of OxLDL. In the present manuscript, we describe the cloning of natural monoclonal antibodies to epitopes of OxLDL from apo E–deficient mice.

## Methods

*Cloning of monoclonal antibodies.* Natural monoclonal antibodies were obtained by fusing B-lymphocytes isolated from the spleens of two female 9-mo-old homozygous apo E–deficient mice with the P3 × 63Ag8.653.1 myeloma cell line by techniques previously described (47). The apo E–deficient mice were from a colony established in La Jolla, CA from breeders provided by Dr. Jan Breslow (Rockefeller University, New York), and were hybrids with a C57BL/6 × 129ola background. These mice were never immunized with an exogenous immunogen. They were fed a high fat diet (88137; Teklad Premier Laboratory Diets, Madison, WI) containing 12.8% milk fat and 0.15% cholesterol (without sodium cholate) for 7 mo. Such dietary intervention induces extensive aortic atherosclerosis, and is accompanied by the formation of high titers of autoantibodies to MDA-lysine (10, 46). Body weights of the two mice at 9 mo were 30.3 and 37.3 g, total plasma cholesterol levels were 1,513 and 2,321 mg/dl, and triglyceride levels were 64 and 260 mg/dl, respectively. To verify the presence of autoantibodies to epitopes of OxLDL, pooled serum from both mice was screened for autoantibodies to native LDL, MDA-LDL, 4-HNE-LDL, and a mixture of 4- and 16-h $Cu^{2+}$-LDL, using immunoassays described below.

Primary screening of supernatants from hybridoma cell lines was performed after 10 d of growth. Hybridomas were selected on the basis of the supernatant's ability to bind native or modified human LDL in solid phase RIAs. Screening antigens included MDA-LDL, 4-HNE-LDL, and a mixture of 4- and 16-h $Cu^{2+}$-LDL generated as previously described (10). Antibody specificities of hybridoma supernatants were determined by solid phase binding and competition RIA (described below). Selected hybridomas were cloned by limiting dilution. Hybridoma cells were injected intraperitoneally into Pristane-primed (Aldrich Chemical Co., Milwaukee, WI) Balb/C mice to produce ascites fluid (47). Immunoglobulin (Ig) subclasses were identified with a commercial isotyping kit (Mallory Laboratories, Springfield, VA). The Ig was isolated by Sepharose 6 HR10/30 chromatography in the presence of 100 mM Tris, 50 mM NaCl, pH 7.8, on a FPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described (48). Fractions containing the Ig were identified immunochemically, concentrated in an Amicon 3050 stirred cell with a YM100 membrane (Amicon Corp., Danvers, MA), and stored at −20°C.

*Preparation of antigens.* Human LDL and HDL were freshly isolated from plasma of healthy human donors by sequential ultracentrifugation in the presence of high concentrations of antioxidants (7). Native LDL was stored at 4°C and used within 2 wk. LDL and other proteins were modified with MDA and 4-HNE as previously described in detail (7). The degree of modification of the lysine residues of apo B was determined by trinitrobenzenesulfonic acid assay (49, 50). The extent of modification was also verified by comparing the electrophoretic mobility of the modified lipoproteins to that of native LDL, using 1% agarose gels (Corning Medical and Scientific, Palo Alto, CA) in borate buffer, pH 8.6 (50, 51). For the initial screening of hybridomas, extensively modified MDA-LDL was used (∼ 75% of the lysine residues modified), whereas MDA-LDL preparations with varying degrees of modification were generated for the subsequent characterization assays. $Cu^{2+}$-LDL was generated by incubating 100 μg LDL/ml PBS, pH 7.35, with 5 μM $CuSO_4$ at 37°C for 1 to 16 h (7), dialyzed against PBS containing EDTA, and concentrated using Centriflo cone-type membrane (CF25; Amicon Corp.). For the early screening of antibodies, a mixture of 4- and 16-h $Cu^{2+}$-oxidized LDL was plated as antigen, to ensure that both early and late oxidation epitopes would be represented.

Modification of LDL or BSA with acrolein and that of BSA with several 2-unsaturated aldehydes was performed as described (51, 52). The degree of LDL modification by acrolein was varied by changing aldehyde concentration, time, and temperature as follows: 20 mM acrolein, 20°C, 1 h; 80 mM acrolein, 20°C, 2 h. BSA was modified with 50 mM acrolein, 50 mM 2-pentenal, 10 mM 2-heptenal, or 3 mM nonenal, for 4 h. In each case, parallel reactions were carried out in the presence of 200 mM $NaCNBH_3$, to reduce Schiff bases (aldimine) to amines, which favors the formation of adducts with the carbonyl group rather than the 3-position carbon. These preparations were labeled "reduced" (R) or "nonreduced" (NR). Excess aldehyde was removed by overnight dialysis at 4°C against Dulbecco's PBS containing 10 μM EDTA.

Reactive products of fatty acid oxidation were also generated by thermal autoxidation of arachidonic acid or linoleic acid. 10 mg arachidonic acid or linoleic acid was transferred to a glass vial open to air and kept at 37°C (arachidonic acid) or 100°C (linoleic acid) for 72 h. The yellow-brown reaction products were dissolved in 50 μl methanol and suspended by vigorous vortexing in 1 ml Dulbecco's PBS with 10 μM EDTA, pH 7.4. Aliquots containing the residue from 1–3 mg oxidized fatty acid were added to 0.5–1 mg LDL or albumin, and brought to a total volume of 1 ml with PBS. The ratio of weight of fatty acid oxidation products to protein was 3 for LDL or albumin adducts moderately modified with arachidonic oxidation products (AOP), and 6 for adducts extensively modified with AOP. The ratio was 6 for adducts of LDL or albumin moderately modified with linoleic oxidation products (LOP), and 12 for adducts extensively modified with LOP. Irreversible derivatization of proteins occurred within minutes at 20°C and was substantially completed within a few hours. After overnight incubation at 20°C, mixtures were dialyzed against PBS containing EDTA. As the lipid peroxidation products in these preparations may interfere with the trintrobenzenesulfonic acid assay, the percentage of lysine residues modified was estimated from the electrophoretic mobility of LDL, using an empirical relationship

based on quantitation of lysine residues by amino acid analysis (51). The extent of modification of AOP- and LOP-modified BSA was not determined for the actual preparations used here, but results of previous studies showed that it was consistently similar to that of LDL modified under identical conditions (51). Acetyl-LDL was generated as previously described (51).

*Determination of autoantibody titers and specificity.* Screening of plasma, hybridoma supernatants, ascites, and purified antibodies was performed with previously described solid-phase RIA techniques (7). For binding assays, 96-well polyvinylchloride microtitration plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 50 μl of antigen (5 μg/ml) in PBS containing 0.27 mM EDTA and 20 μM butylated hydroxytoluene overnight at 4°C. Non adherent antigen was aspirated and remaining binding sites were "blocked" by incubation with 2% BSA (RIA grade, Sigma Chemical Co., St. Louis, MO) in PBS for 45 min at room temperature. The wells were washed four times with PBS containing 0.27 mM EDTA, 0.02% $NaN_3$, 0.05% Tween 20, and 0.001% aprotinin (washing buffer), using a microtiter plate washer. Serial dilutions of antibody in washing buffer containing 3% BSA (dilution buffer) were prepared, added at 50 μl/well, and incubated overnight at 4°C. The amount of immunoglobulin bound was quantitated with affinity-purified goat anti mouse IgG or IgM (Sigma Chemical Co.) labeled at 3,000–12,000 cpm/ng with $^{125}I$ using lactoperoxidase (Enzymobeads; Bio-Rad Laboratories, Richmond, CA). Plates were incubated for 4 h at 4°C with 50 μl/well of the secondary antibody, diluted to 400,000 cpm/50 μl dilution buffer.

In initial studies, sera from the two apo E-deficient mice used to generate the monoclonal antibodies and from two age-matched C57BL/6 mice were screened for circulating autoantibodies against selected antigens, using the above described binding assay. For these studies, a titer was defined as the reciprocal of the highest dilution that gave binding to the antigen three times higher than binding to BSA. For subsequent studies, the actual dilution curves are displayed (e.g., Fig. 9).

Classical competitive solid-phase RIAs were performed as described (7), except that the antigen was plated at 1 μg/ml. A fixed and limiting amount of the natural monoclonal antibody (25 μl) was added together with an equal volume of dilution buffer containing increasing amounts of competitors. Results were calculated as $B/B_0$; i.e., the amount of antibody bound to the plated antigen in the presence of competitor (B) divided by the binding in the absence of competitor ($B_0$). Alternatively, in some competition assays, the purified monoclonal antibodies (i.e., E04 and E014) were directly radiolabeled with $^{125}I$ as described above, and competition was determined by directly measuring binding of the monoclonal antibody to the plated antigen in the absence or presence of unlabeled competitors, including other unlabeled purified monoclonal antibodies.

Titers of autoantibodies in human sera were determined using a chemiluminescent detection system. In this assay, 5 μg/ml of the antigen in 50 mM Tris-buffered saline (TBS) was added to each well of a 96-well FluoroNunc plate (Nunc Inc., Naperville, IL), and incubated for 2 h at 4°C. Plates were washed as described for the RIA, and serial dilutions of human sera in TBS containing 3% BSA were added and incubated for 1 h at room temperature. After a thorough wash, plates were incubated with 50 μl/well of a 1:30,000 dilution of alkaline-phosphatase–labeled goat anti–human IgG or IgM for 1 h at room temperature. Plates were washed with TBS containing 0.05% Tween 20, and then with distilled water. 25 μl of a 20% solution of Lumi-Phos 530 (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added to each well, and plates were incubated for 1 h at room temperature in the dark. Luminescence was then determined using a Lucy 1 luminometer and WINLCOM software (Anthos Labtec Instruments, Salzburg, Austria). Data were expressed as number of flashes of light in 100 ms.

*Immunocytochemistry.* Selected natural monoclonal antibodies were used to immunostain atherosclerotic lesions of LDL receptor–deficient rabbits, balloon-catheterized, cholesterol-fed New Zealand white (NZW) rabbits, and human brain arteries obtained at autopsy. For comparison, previously generated monoclonal antibodies MDA2, specific for MDA-lysine (7), and NA59, specific for 4-HNE-lysine, were used. Smooth muscle cells in human lesions were identified by immunocytochemistry with HHF35, a monoclonal antibody recognizing alpha and gamma actin (Enzo Diagnostics, Inc., Farmingdale, NY) (53). Macrophage-derived cells in human lesions were stained with HAM56 (Enzo Diagnostics) (54). Tissues were obtained as de-

*Table I. Number of Original Hybridoma Cell Lines Secreting Antibodies to Epitopes of OxLDL*

| Antigens recognized | Positive pooled supernatants |
|---|---|
| | *n* |
| MDA-LDL only | 198 |
| $Cu^{2+}$-LDL only | 10 |
| 4-HNE-LDL only | 5 |
| MDA-LDL and $Cu^{2+}$-LDL | 32 |
| MDA-LDL and 4-HNE-LDL | 86 |
| $Cu^{2+}$-LDL and 4-HNE-LDL | 5 |
| MDA-LDL, 4-HNE-LDL, and $Cu^{2+}$-LDL | 158 |
| Total | 494 |

The initial cloning yielded 1,536 cell lines. Supernatants from two wells were pooled after 10 d and tested for binding to native LDL, MDA-LDL, $Cu^{2+}$-LDL, and 4-HNE-LDL in a solid-phase radioimmunoassay. Positive wells were defined as a ratio of binding to modified LDL divided by binding to native LDL greater than three (10, 19). Of the 768 pooled samples, 494 were positive for one or more epitopes of OxLDL.

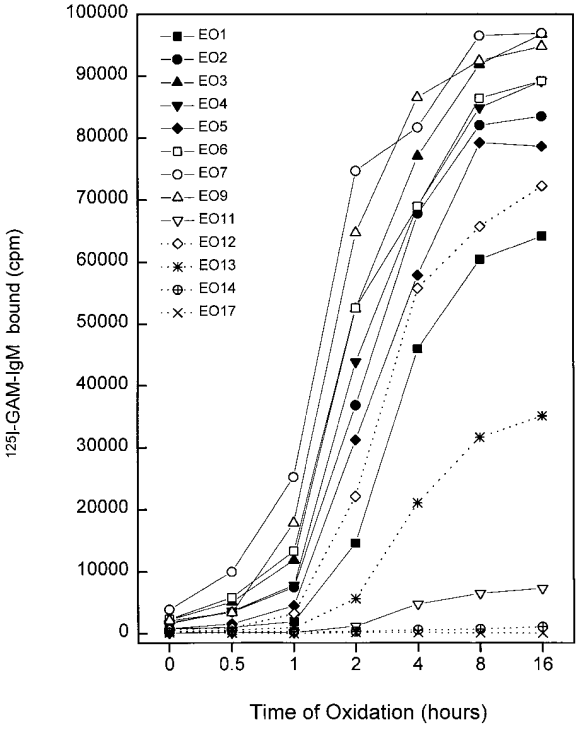



*Figure 1.* Binding of natural monoclonal antibodies to LDL oxidized to different extents. Native LDL (0 time) was exposed to copper ions (5 μM) for indicated periods of time and then used to coat microtiter wells. 50 μl of each purified antibody were added per well, and antibody binding detected with $^{125}I$-labeled goat anti–mouse IgM. Note that the time scale is not linear.

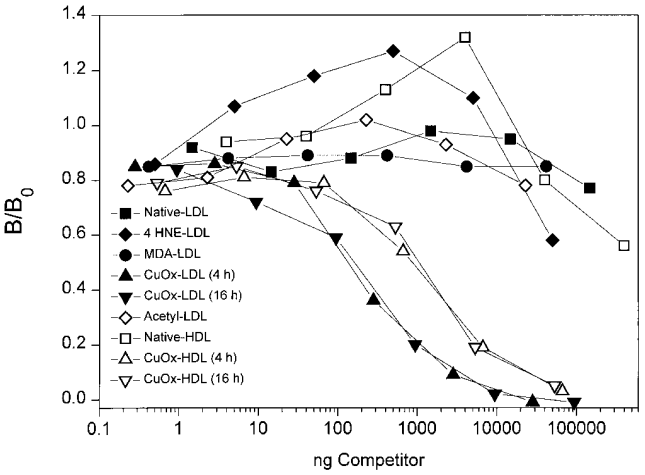



*Figure 2.* Competitive RIA with the natural monoclonal antibody E04, originally selected for its binding to copper-oxidized LDL, using various forms of modified LDL and HDL. LDL oxidized with copper for 16 h was plated at 1 μg/ml, and a fixed concentration of $^{125}$I-labeled antibody E04 was added together with increasing concentrations of competitor. Results are expressed as binding of $^{125}$I-labeled E04 to the plated antigen in the presence of competitor ($B$) divided by binding in the absence of competitor ($B_o$). Copper oxidized (CuOx)-LDL and CuOx-HDL, copper-oxidized human LDL and HDL, respectively (the parentheses indicate the length of the time of incubation with copper); acetyl-LDL, human LDL modified with acetyl.

scribed (6, 21), fixed in formal sucrose (4% paraformaldehyde, 5% sucrose) (rabbit arteries) or 10% formaldehyde (human arteries) and paraffin-embedded. Serial sections (8μm thick) were rehydrated and immunostained using an avidin-biotin-alkaline phosphatase system (Vector Laboratories, Inc., Burlingame, CA), as previously described (10, 21, 55). Primary antibodies bound to the tissue were detected with biotinylated anti–mouse immunoglobulin serum (Vector Laboratories, Inc.). Control slides were incubated without primary antibody.

## Results

*Cloning of monoclonal autoantibodies.* Natural monoclonal antibodies were generated from two nonimmunized female 9-mo-old apo E–deficient mice after 7 mo on a high fat diet. Sera from these two animals obtained at the time of sacrifice were pooled and screened for autoantibodies to $Cu^{2+}$-LDL, MDA-LDL, 4-HNE-LDL, and native LDL in solid-phase immunoassays with heavy chain–specific second antibodies. As expected, the pooled sera contained high titers of autoantibodies binding to MDA-LDL, predominantly IgG and IgM (titers $> >$ 5,000). In addition, high titers of autoantibodies to 4-HNE-LDL and $Cu^{2+}$-LDL ($>$ 5,000), but not to native LDL were found (data not shown). No significant titers of IgA antibodies to these epitopes were detected.

Generation of hybridomas depends on actively proliferating B cells. At the time of sacrifice, the spleens of the two apo E–deficient mice from which the B cells were obtained were considerably larger and heavier than those of age-matched C57BL/6 mice. The same was true when the weights of the spleens were adjusted for the body weights (33.4 vs 25.0 mg/g body wt in apo E–deficient and C57BL/6 mice, respectively). Presumably, this indicates the presence of a large number of proliferating B cells in the apo E–deficient mice. Monoclonal antibodies were then generated as described in Methods. The fusions yielded 1,536 wells with viable hybridoma cell growth. Supernatants from these wells were initially pooled (two each) and screened against the four antigens listed above. The first screening revealed a surprisingly large number of supernatants containing antibodies that bound to epitopes of OxLDL (Table I). No antibodies were found binding exclusively to native LDL, though several bound to both native LDL and one or more forms of oxidized LDL. Of the 768 pooled samples tested, 64% were positive; i.e., showed binding to at least one form of OxLDL that was three fold greater than binding to native LDL. In other words, the percentage of original clones secreting antibodies to OxLDL was between 32 and 64%. Hybridoma cell lines contributing to the positive wells were then assayed individually, and hybridomas showing the greatest antibody binding were selected for cloning by limiting dilution.

After cloning, a panel of 13 monoclonal antibodies (designated "E0" antibodies) was characterized in greater detail. All had been selected for recognition of either $Cu^{2+}$-LDL or MDA-LDL. One particular antibody, E011, was selected because it originally showed greater binding to native LDL than to oxidized LDL. However, it should be stressed that E011 is not specific for native LDL. Unfortunately, none of the cell lines initially selected for recognition of 4-HNE-LDL survived or continued to secrete sufficient amounts of immunoglobulin. All 13 monoclonal antibodies were isotyped as IgM, and were shown to be unique by isoelectric focusing.

*Characterization of antibodies.* We first compared the binding of the 13 antibodies to progressively $Cu^{2+}$-oxidized LDL. In general, antibodies initially selected for predominant recognition of $Cu^{2+}$-LDL (E01 to E09) recognized epitopes that were progressively formed during the first 4 h of oxidation of LDL (Fig. 1). For many of the antibodies, the binding reached a plateau with LDL oxidized between 8 and 16 h. None of the antibodies bound to epitopes of OxLDL present exclusively in LDL oxidized for only short time periods. However, it should be remembered that LDL oxidized for 4 and 16 h had been used as the screening antigen. Antibodies initially selected primarily for their binding to "native" LDL (E011) or MDA-LDL (E013 to E017) bound poorly to this preparation of $Cu^{2+}$-LDL, with the exception of E012 and E013. Although some MDA-lysine epitopes are formed during $Cu^{2+}$-oxidation (7), the strong binding of E012 and E013 suggested that these antibodies may recognize an epitope immunologically related to but structurally different from MDA-lysine. However, differences in antibody affinity towards MDA-lysine could not be ruled out as a potential explanation. Similar experiments with different LDL preparations revealed considerable variability in the recognition of the progressively oxidized LDL by the E0 antibodies, in particular the recognition of native and mildly oxidized LDL.

Further characterization of the newly generated E0 monoclonal antibodies by competitive RIAs demonstrated that the specificity of some of these natural antibodies closely resembled that of monoclonal antibodies to MDA-LDL and $Cu^{2+}$-LDL we previously generated by immunizing mice with murine LDL modified in vitro (6, 7). One such example is E04, originally selected for its binding to $Cu^{2+}$-LDL (Fig. 2). In this experiment, E04 was purified from ascites, radiolabeled, and used at a limiting dilution in a competitive RIA with 16 h $Cu^{2+}$-oxidized LDL as plated antigen. Complete competition was achieved by 4 and 16 h $Cu^{2+}$-oxidized LDL, as well as by 4 and 16 h $Cu^{2+}$-oxidized HDL, indicating that the epitope rec-

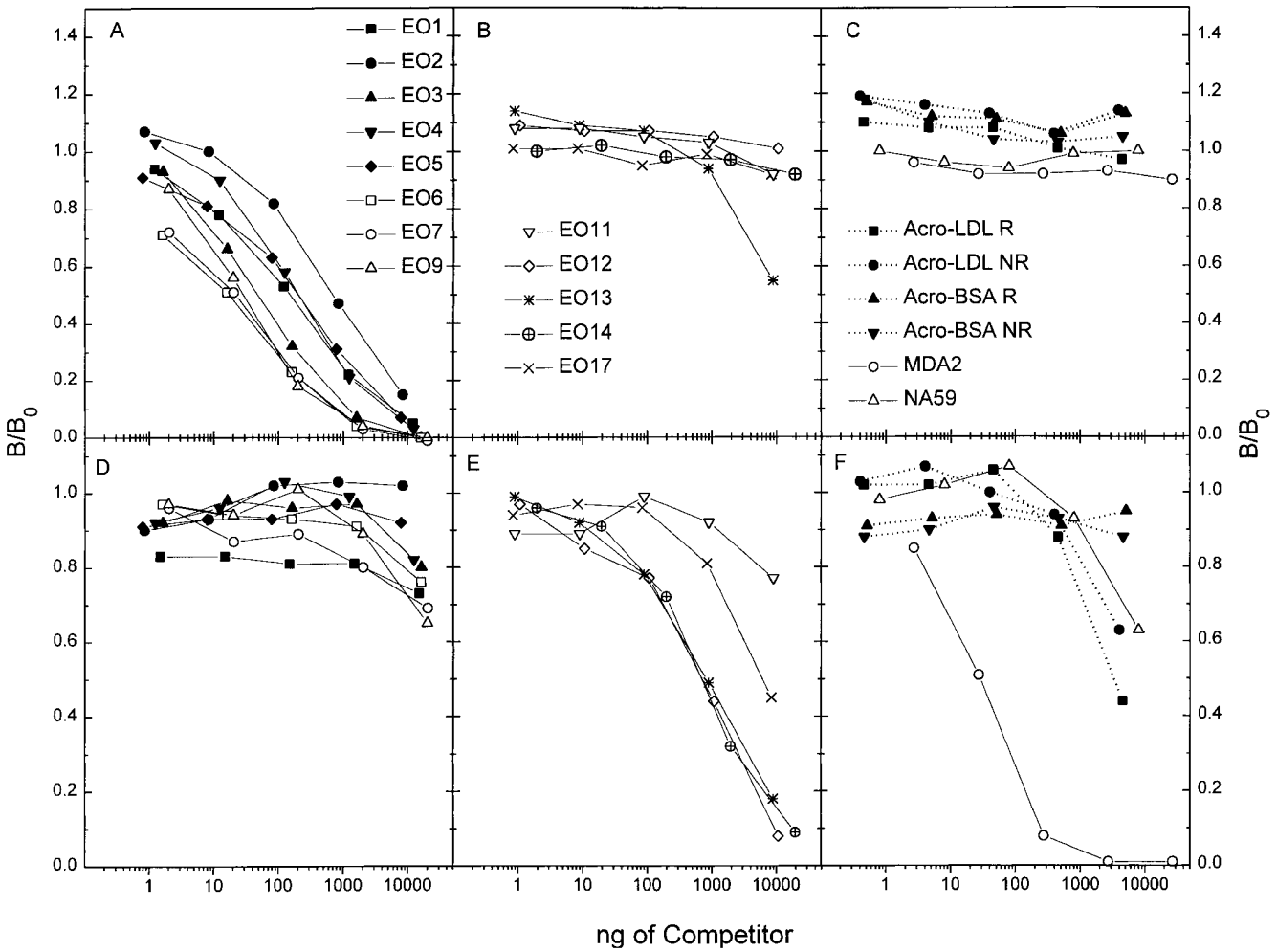



*Figure 3.* (*A–C*) Ability of natural monoclonal antibodies and modified proteins to compete with E04 for binding to $Cu^{2+}$-LDL. $Cu^{2+}$-LDL was plated as antigen (1 μg/ml), and 25 μl (75,000 cpm) of $^{125}$I-labeled antibody E04 (~ 4,000 cpm/ng) was added together with indicated concentrations of unlabeled competitors. Results are expressed as $B/B_0$, as described in Fig. 2. Competition by monoclonal antibodies originally selected for binding to $Cu^{2+}$-LDL is shown in *A*, that by monoclonal antibodies selected for binding to MDA-LDL or native LDL in *B*. Competition by acrolein-modified proteins and by previously generated monoclonal antibodies MDA2 (specific for MDA-lysine) and NA59 (specific for 4-HNE-lysine) is shown in *C*. Acro LDL NR and Acro LDL R, LDL extensively modified with 80 mM acrolein under nonreducing and reducing conditions, respectively; Acro BSA NR and Acro BSA R, BSA modified with acrolein under nonreducing and reducing conditions, respectively. (*D–F*) Ability of natural monoclonal antibodies and modified proteins to compete with E014 for binding to MDA-modified LDL. Extensively modified MDA-LDL was plated as antigen (1 μg/ml), and 25 μl (75,000 cpm) of $^{125}$I-labeled antibody E014 was added together with the indicated concentrations of unlabeled competitors.

ognized by E04 is not specific for modified LDL alone. Thus, the specificity of E04 resembles that of OLF4-3C10, an induced monoclonal antibody previously generated by immunizing mice with apoprotein fragments of 4 and 18 h $Cu^{2+}$-oxidized murine LDL (7, 21).

We then tested the ability of the other E0 monoclonal antibodies to compete with labeled E04 for binding to $Cu^{2+}$-LDL (Fig. 3, *A–C*). All antibodies originally selected for binding to $Cu^{2+}$-LDL were able to effectively compete with E04 for binding to the plated antigen (Fig. 3 *A*). The slopes of the competition curves were parallel, indicating the same relative affinity for the epitope(s) recognized. However, antibodies E01 to E09 were genuinely different and not just clones of the same antibody, as established by unique isoelectric focusing patterns (data not shown). In contrast, none of the natural monoclonal antibodies originally selected for binding to MDA-LDL or native LDL competed, with the exception of E013 (Fig. 3 *B*). This is consistent with the ability of E013 to bind to $Cu^{2+}$-LDL, as well as MDA-LDL. MDA2 and NA59, two previously generated monoclonal antibodies specific for MDA-lysine and 4-HNE-lysine, respectively, did not compete with E04, demonstrating that the epitope of $Cu^{2+}$-LDL recognized by E04 is different from either of these two forms of lysine modification (Fig. 3 *C*).

In a similar manner, we characterized another natural antibody, E014, originally selected for binding to MDA-LDL (Fig. 3, *D–F*). In this competitive RIA, MDA-LDL was plated as antigen. As expected, none of the natural monoclonal antibodies originally selected for binding to $Cu^{2+}$-LDL (E01–E09) was an effective competitor of E014 (Fig. 3 *D*). By contrast, monoclonal antibodies E012, E013, and E017, as well as E014 itself, were effective competitors (Fig. 3 *E*). E011, the antibody originally selected for binding to native LDL competed poorly (Fig.

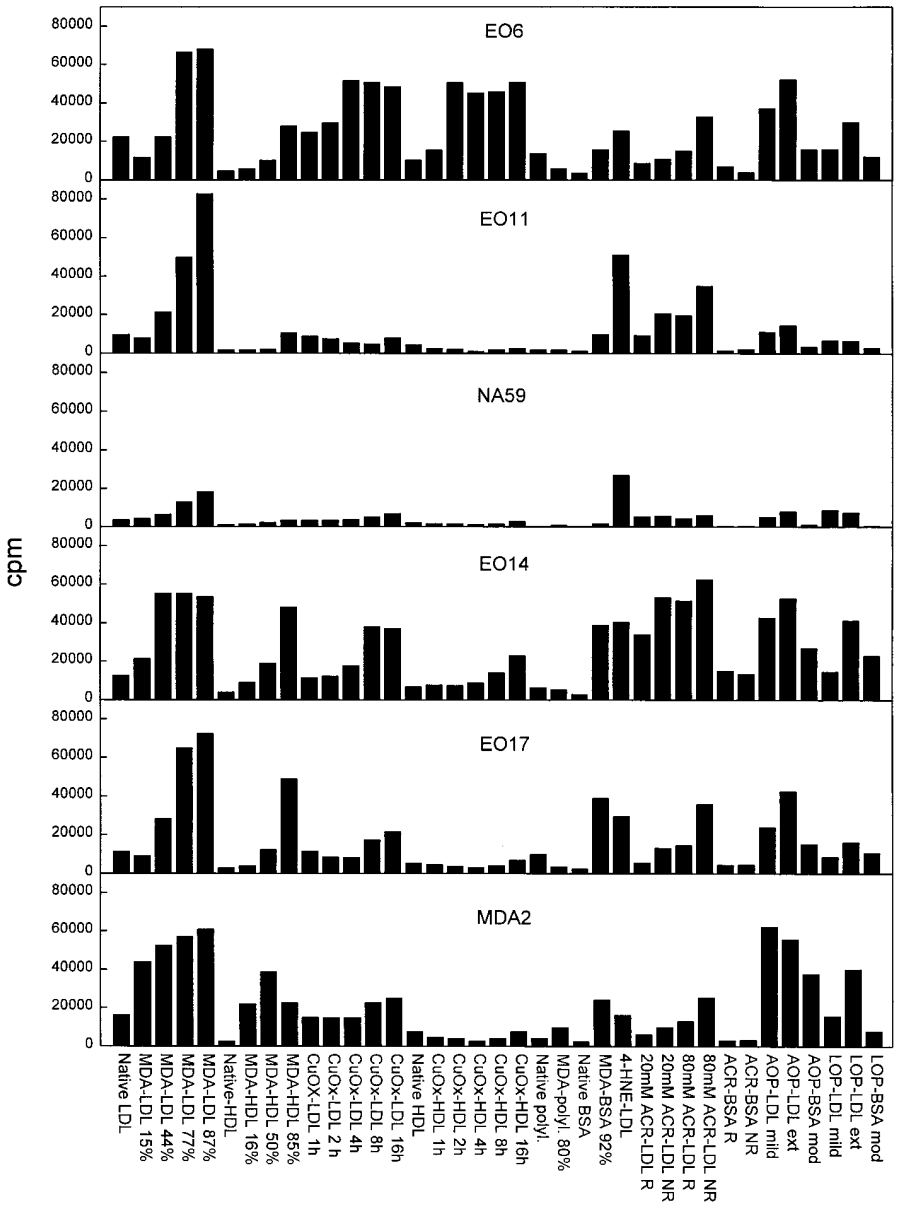



*Figure 4.* Binding of representative natural monoclonal antibodies to various native and modified antigens. Each antigen was plated at 5 μg/ml, and equal amounts of purified antibodies (10 μg/ml) were added per well. Antibody binding was detected with $^{125}$I-labeled goat anti–mouse IgM. In this experiment, no correction for nonspecific binding to the plated antigens was made, and results are reported as cpm bound (note however, the low level of binding to BSA). The entire panel of natural monoclonal antibodies was tested for binding to these and other antigens under identical conditions. Antibodies shown in the figure were either typical of a group of antibodies selected with the same screening antigen, or clearly recognized a different panel of antigens in this assay. See text for a detailed explanation of results. MDA-HDL, high density lipoprotein modified with MDA; MDA-BSA, BSA modified with MDA; CuOx-LDL (or -HDL), LDL (or HDL) oxidized with 5 μM copper. For these preparations, the numbers refer to the extent of lysine modification or to the time of oxidation. Acro LDL (or BSA) NR and Acro LDL (or BSA) R, LDL (or BSA) modified with acrolein under nonreducing or reducing conditions (the concentration refers to the amount of acrolein used); AOP-LDL (or BSA), LDL (or BSA) extensively modified by arachidonic acid oxidation products; LOP-LDL (or BSA), LDL (or BSA) extensively modified by linoleic acid oxidation products.

3 *E*). MDA2, the induced monoclonal antibody specific for MDA-lysine, was a very efficient competitor for the binding of E014 to the plated antigen (Fig. 3 *F*). NA59 also competed to some extent. Thus, these preliminary studies suggested that the specificity of E014 resembled that of MDA2.

*Recognition of other forms of OxLDL.* To begin to further define the nature of the epitopes recognized by the natural monoclonal antibodies, they were screened with other forms of oxidatively modified lipoproteins and proteins, based on model epitopes described by Zhang et al., (51) and Steinbrecher et al. (52) (Fig. 4). These included LDL and BSA modified with (*a*) different concentrations of acrolein under non reducing and reducing conditions, (*b*) arachidonic and linoleic acid oxidation products, and (*c*) aldehydes such as 4-hydroxynonenal, 2-pentenal, or 2-heptenal. Preparations of MDA-LDL, in which an increasing percentage of lysine residues were modified, and LDL and HDL progressively oxidized with copper were also used in the same assay. In these experiments, a constant amount of antigen (5 μg/ml) was plated, and a constant amount of the purified antibody (10 μg protein/ml) was added to each well.

Most of the antibodies originally selected for their recognition of $Cu^{2+}$-LDL displayed similar binding patterns as E06, and bound well to epitopes of $Cu^{2+}$-LDL and $Cu^{2+}$-HDL (Fig. 4). E06 did not recognize MDA-LDL, in which 44% of lysines were modified, but did bind to very extensively modified MDA-LDL, presumably due to generation of other oxidation epitopes in these preparations. E06 also bound well to LDL modified by arachidonic acid and, to a lesser extent, LDL modified by linoleic acid oxidation products, and to acrolein-modified LDL. As shown in the companion paper (32), E06 bound prominently to epitopes of oxidized phospholipids and oxidized phospholipid-protein adducts.

E011, the antibody originally selected for its binding to native LDL, showed strong recognition of both 4-HNE-LDL and heavily modified MDA-LDL, and resembled in many aspects the monoclonal antibody NA59 previously generated by immunization with 4-HNE-LDL.

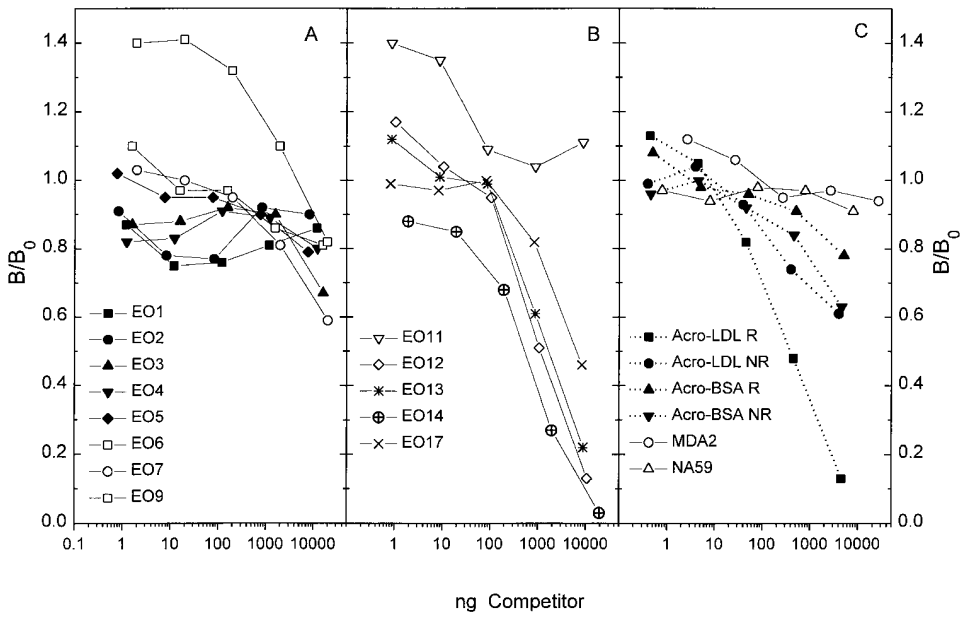



*Figure 5.* Ability of natural monoclonal antibodies and various forms of native and modified proteins to compete with E014 for binding to acrolein-LDL. Acrolein-LDL, prepared under reducing conditions, was plated as antigen (1 μg/ml), and 25 μl of $^{125}$I-labeled antibody E014 was added together with indicated concentrations of competitors. Competition by natural monoclonal antibodies originally selected for binding to copper-oxidized LDL is shown in *A*, and by antibodies selected for binding to MDA-LDL or native LDL in *B*. Competition by acrolein-modified proteins and monoclonal antibodies MDA2 (specific for MDA-lysine) and NA59 (specific for 4-HNE-lysine) is shown in *C*. Acro LDL (or BSA) NR and Acro LDL (or BSA) R, LDL (or BSA) extensively modified with 80 mM acrolein under nonreducing and reducing conditions, respectively.

Antibodies originally selected for binding to MDA-LDL showed less uniform binding patterns (Fig. 4). E013 (data not shown), E014, and E017 recognized MDA-LDL and MDA-HDL. More extensively modified forms of $Cu^{2+}$-LDL were also recognized, but $Cu^{2+}$-HDL was not recognized by E013 and E017 and only poorly by E014. Furthermore, E014 and E017 bound to reduced and nonreduced acrolein-modified LDL, with a preference for the nonreduced form, and to 4-HNE-LDL. However, the relative binding to these antigens, as well as the recognition of LDL modified by arachidonic or linoleic acid oxidation products was different for each antibody. E013, the antibody that had displayed an atypical recognition of $Cu^{2+}$-LDL, showed particularly good binding to acrolein-LDL (data not shown). E014 (and E012, data not shown) recognized both moderately modified MDA-LDL and acrolein-LDL, and also bound to LDL and BSA modified by fatty acid peroxidation products. It is of interest that MDA2, which specifically binds MDA-lysine (7), also showed strong binding to LDL modified by arachidonic or linoleic acid oxidation products, and some binding to acrolein-modified LDL as well. Presumably, in addition to MDA, MDA-like structures, such as adducts formed with the 3-carbon aldehyde acrolein, are recognized as well, suggesting that the epitope recognized is more complex than simply MDA-lysine. None of the E0 monoclonal antibodies showed significant binding to LDL or BSA modified with 2-pentenal or 2-heptenal (data not shown).

Recognition of acrolein-modified LDL and BSA prepared under nonreducing and reducing conditions was studied in even greater detail for E04 and E014. As shown in Fig. 3 *C*, acrolein-modified proteins did not compete for binding of radiolabeled E04 to $Cu^{2+}$-LDL. By contrast, acrolein-modified proteins showed some competition for binding of E014 to heavily modified MDA-LDL (Fig. 3 *F*). Fig. 5 represents a similar competition assay with radiolabeled E014, using LDL modified with 80 mM acrolein under nonreducing conditions as plated antigen. The E0 antibodies originally selected for binding to $Cu^{2+}$-LDL competed poorly with E014 for binding to acrolein-LDL (Fig. 5 *A*). Natural monoclonal antibodies originally selected for binding to MDA-LDL (E012, E013, and E017), which had shown binding to acrolein-modified LDL in Fig. 4 and of course E014 itself, competed effectively, indicating that they recognize the same epitope in acrolein-LDL as E014 (Fig. 5 *B*). By contrast, E011, which also bound to acrolein-LDL (Fig. 4), did not compete with E014, indicating that this antibody recognizes a different epitope. Even though monoclonal antibody MDA2 competed for binding of E014 to MDA-LDL (Fig. 3 *F*), it did not compete with E014 for binding to acrolein-LDL (Fig. 5 *C*). Thus, the exact epitopes on acrolein-LDL recognized by E014 and MDA2 are different. As expected, acrolein-modified LDL was an effective competitor (Fig. 5 *C*).

*Immunocytochemistry with natural monoclonal antibodies.* The differences in specificity between the cloned autoantibodies established by binding and competition assays may indicate that these antibodies see different parts of the same structure (or of immunologically related structures). In this case, one would expect that the distribution of epitopes recognized in atherosclerotic lesions would be the same; i.e., that immunocytochemistry with different antibodies would yield similar staining patterns. Alternatively, the antibodies might recognize independent antigens that may predominate in certain stages of lesion development, or at particular sites within the lesion. To address this question, and to confirm the in vivo occurrence of the epitopes recognized, immunocytochemistry with the entire panel of E0 monoclonal antibodies was performed on serial sections of atherosclerotic lesions of various stages and of different composition. Atherosclerotic lesions were studied in arteries from LDL receptor-deficient rabbits, NZW rabbits in which lesion formation had been induced by balloon catheterization and feeding of a cholesterol-rich diet, and in brain arteries from human subjects obtained at autopsy. Each of the 13 natural monoclonal antibodies immunostained atherosclerotic lesions. Fig. 6 shows representative examples of the immunostaining. In general, the distribution of epitopes

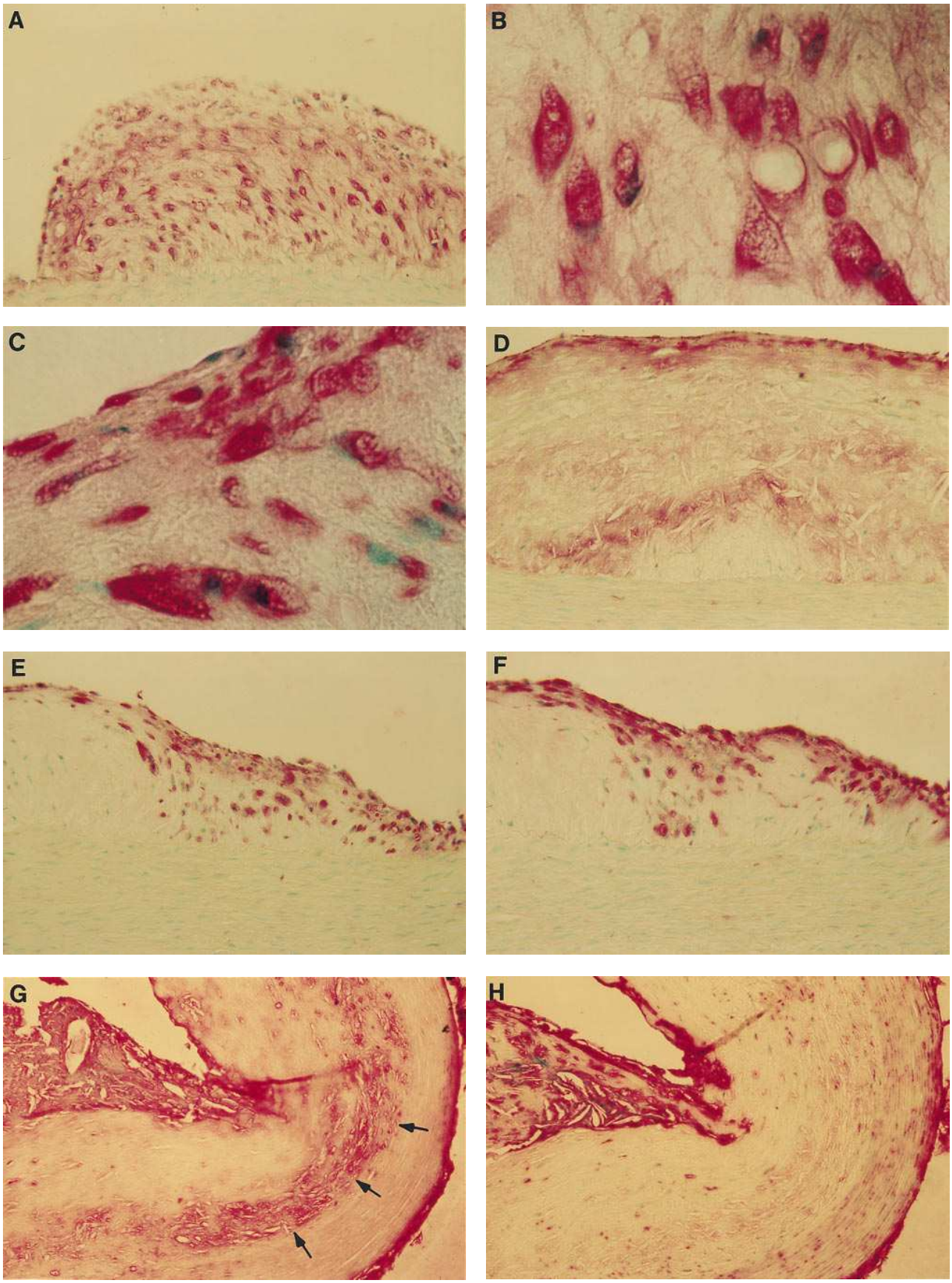



*Figure 6.* Immunocytochemistry of atherosclerotic lesions from the aorta of LDL receptor-deficient rabbits or balloon-catheterized NZW rabbits fed a high cholesterol diet, using several of the natural monoclonal antibodies. Sections were prepared as described in Methods, and stained

recognized by our panel of E0 antibodies resembled that previously obtained with induced monoclonal antibodies to OxLDL such as MDA2 and NA59 (6, 21). In early atherosclerotic lesions from both cholesterol-fed NZW and LDL receptor-deficient rabbits, staining was predominantly foam-cell associated (Fig. 6, *A–C*), although diffuse extracellular staining was also observed in some areas. More advanced lesions showed "oxidation-specific" epitopes in the shoulder regions and in the cap, as well as diffuse staining in the necrotic core (Fig. 6 *D*). Normal arteries showed no intimal staining with the natural monoclonal antibodies, and control sections in which the primary antibody was omitted were devoid of any staining (data not shown).

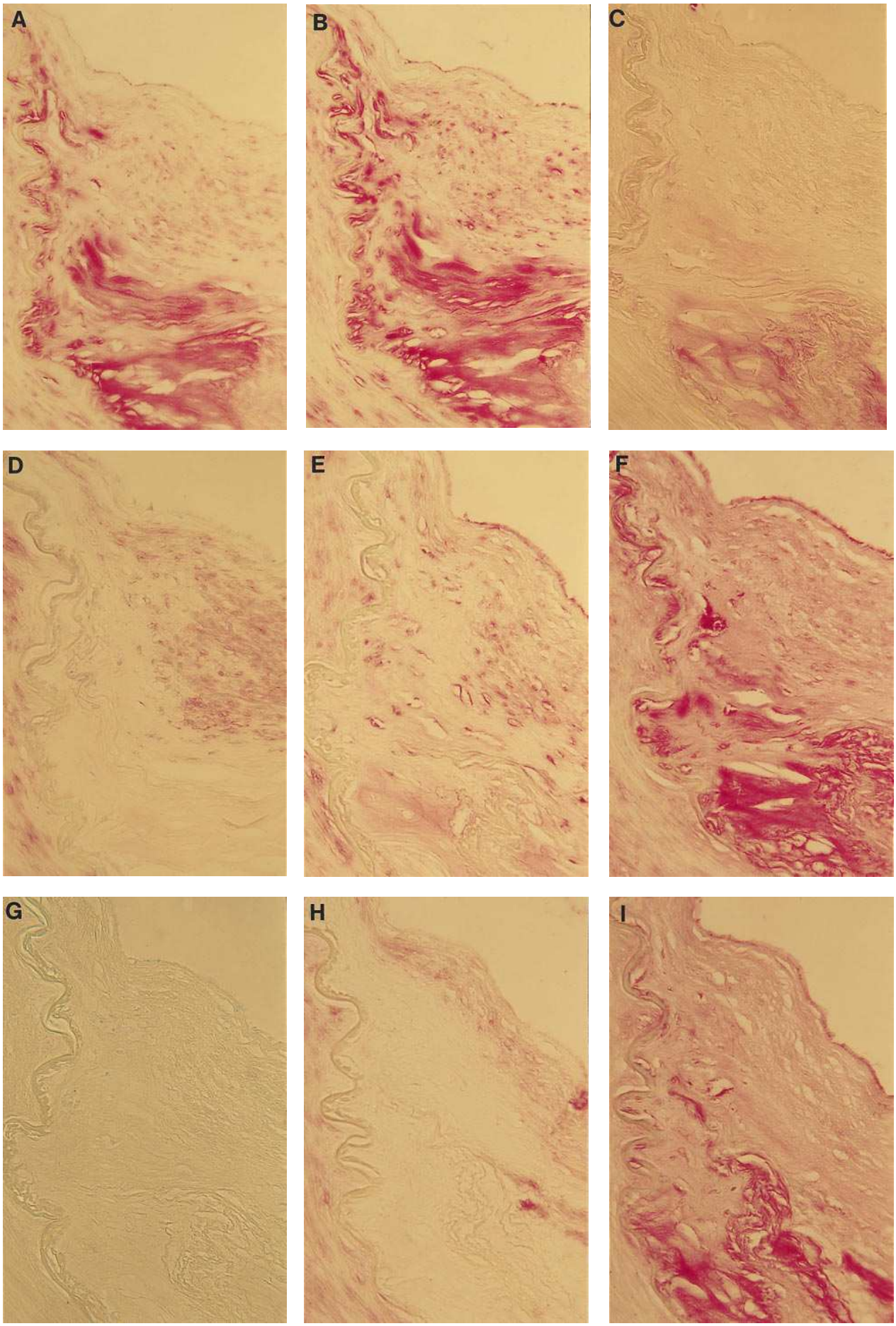
In most of the early and transitional lesions examined to date, the distribution of immunostaining obtained with natural monoclonal antibodies of different specificity was very similar (Fig. 6, *E* and *F*). However, in some very advanced lesions from LDL receptor-deficient rabbits, significant differences were found even between antibodies originally selected for binding to the same epitope. For example, an area adjacent to the internal elastic lamina in the very advanced lesion shown in Fig. 6, *G* and *H* was rich in epitopes recognized by E04 and most other natural monoclonal antibodies (*arrows*), but not by E017.

Immunocytochemistry was therefore extended to very advanced atherosclerotic lesions found in human brain arteries. In contrast to the rabbit lesions, which had been perfusion fixed in the presence of high concentrations of antioxidants to exclude oxidative artifacts, the human specimens were obtained postmortem, and the staining therefore may not accurately reflect the extent of lipoprotein oxidation occurring in vivo. These human arteries were used only to compare the natural and induced monoclonal antibodies to OxLDL. Fig. 7 shows sections of the shoulder area of a classical atheroma stained with natural and induced monoclonal antibodies, and reveals that some of the natural monoclonal antibodies show distinct staining patterns. In general, staining was most intense in a necrotic area, although diffuse staining was found throughout the core region. Macrophage- and smooth muscle cell–derived foam cells in the shoulder area and cap also stained, and some degree of staining was even seen in the media. The natural monoclonal antibody E04 (Fig. 7 *A*) yielded virtually identical staining patterns as E01 and E03 (not shown). Staining with E06 (Fig. 7 *B*) was also very similar, with both cellular and extracellular staining, in particular in the core. In contrast, E011 (Fig. 7 *C*) recognized epitopes almost exclusively in the core, and showed almost no cellular staining in the cap. E014 (Fig. 7 *D*) also showed a unique staining pattern in this particular lesion, and recognized epitopes almost exclusively found in the cell-rich shoulder area and cap. E017 (Fig. 7 *E*) showed relatively weak staining in both cell-rich and necrotic areas, but lacked the predominance of the staining in the necrotic core. Two subsequent experiments using greater concentrations of E014 did not yield stronger staining. Surprisingly, immunostaining with MDA-2, the monoclonal antibody generated with MDA-LDL (Fig. 7 *F*), was more similar to that obtained with natural monoclonal antibodies originally selected for binding to $Cu^{2+}$-LDL than to that of E014 and E017, originally selected for binding to MDA-LDL. These results not only confirm the differences in specificity between the natural monoclonal antibodies, but also suggest that specific epitopes may be formed during different stages of lesion development and/or at certain sites within lesions, or that conservation of these epitopes occurs differentially at different sites. Again, control sections stained without primary antibody were devoid of any staining (Fig. 7 *G*).

*Screening of murine sera for autoantibody populations.* To determine if any of the novel epitopes recognized by our panel of cloned monoclonal antibodies would also be recognized by circulating antibodies, we used acrolein-modified LDL and BSA, as well as LDL modified by arachidonic and linoleic acid oxidation products to screen sera of apo E–deficient mice and C57BL/6 mice for autoantibodies. Binding curves from pooled sera for IgM autoantibodies are shown in Fig. 8. The original screening antigens ($Cu^{2+}$-LDL, MDA-LDL, 4-HNE-LDL) were also used in the same assays for comparison. In sera from apo E–deficient mice, the titer of IgM autoantibodies to MDA-LDL was the highest, followed by the titers to $Cu^{2+}$-LDL and 4-HNE-LDL (Fig. 8 *A*). Autoantibody titers to nonreduced or reduced acrolein-LDL and AOP-LDL were comparable to those against $Cu^{2+}$-LDL and 4-HNE-LDL (Fig. 8 *B*). Somewhat lower titers were also present against LOP-LDL. In contrast, sera from C57BL/6 mice contained measurable autoantibody titers only to MDA-LDL (Fig. 8 *C*). Results for IgG autoantibodies were essentially identical to those obtained for IgM autoantibodies (data not shown).

*Screening of human sera for autoantibodies to acrolein-modified LDL.* Sera from six human subjects were also screened for IgG and IgM autoantibodies to acrolein-modified LDL, and, for reasons of comparison, for autoantibodies to MDA-LDL and native LDL. All human sera had autoantibodies to acrolein-LDL comparable to those against MDA-LDL (titers between 1,000 and 10,000). Although the number of sera tested was too small for a statistical evaluation, it is notewor-

with the avidin–biotin–alkaline phosphatase method. The presence of the antigen recognized by the primary antibody is indicated by a red substrate; nuclei were counterstained with methyl green. (*A*) Macrophage/foam cell–rich lesion from an NZW rabbit stained with E04 (dilution 1:500). (*B*) Greater magnification of a serial section stained with E02 (dilution 1:100) demonstrating that the epitopes recognized are predominantly cell associated. (*C*) Shoulder area of a transitional lesion from an LDL receptor-deficient rabbit stained with E02, also showing predominantly macrophage-associated staining. (*D*) Advanced atherosclerotic lesion of an LDL receptor-deficient rabbit with an extensive necrotic core containing cholesterol crystals. Staining with E06 (dilution 1:100) is found in superficial macrophages as well as extracellularly, in the core region. (*E* and *F*) Serial sections of a transitional lesion of an LDL receptor-deficient rabbit stained with E04 (*E*), which recognizes primarily $Cu^{2+}$-oxidized lipoproteins, and with E06 (*F*), which recognizes a modified phospholipid epitope. In this type of lesion, the distribution of epitopes recognized by all natural monoclonal antibodies was very similar. (*G* and *H*) In contrast, the staining pattern differed in other lesions, such as this very advanced atheroma from an LDL receptor-deficient rabbit. Most of the antibodies originally selected for recognition of copper-oxidized LDL (e.g., E04, *G*) or malondialdehyde-LDL (data not shown) recognized epitopes both in the superficial, macrophage-rich area of this lesion and in a deeper region adjacent to the internal elastic lamina (*arrows*), whereas the latter area was relatively devoid of staining by E017 (*H*). *A*, *D–F*, ×139; *B* and *C*, ×696; *G* and *H*, ×87.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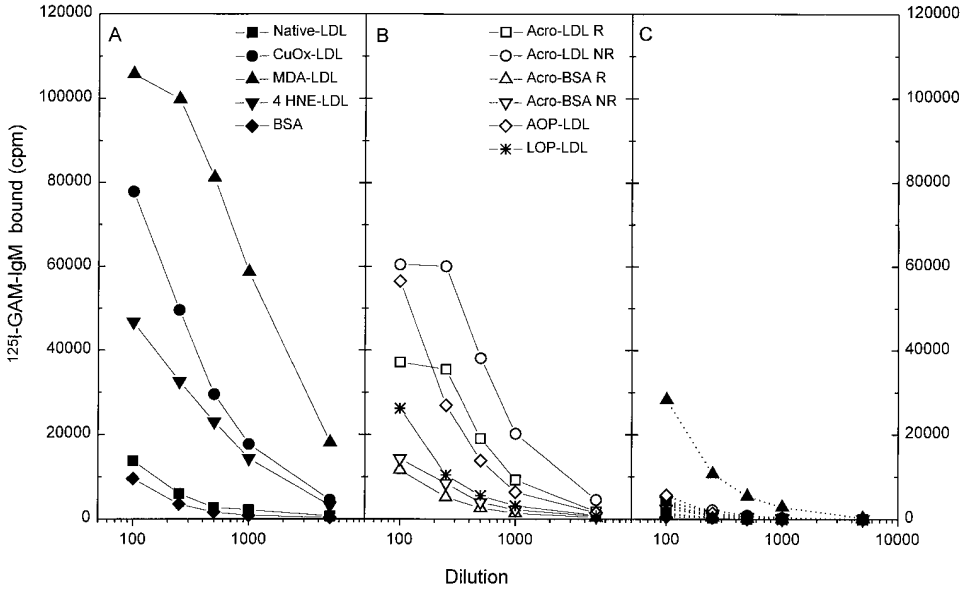


*Figure 8.* Dilution curves of IgM autoantibodies in the pooled sera of the two apo E–deficient mice used to generate the E0 monoclonal antibodies (*A* and *B*), and in the pooled sera of two age-matched C57BL/6 mice fed regular mouse chow (*C*). All antigens were plated at 5 μg/ml, and serum autoantibodies bound were detected with $^{125}$I-labeled GAM-IgM, as described in Methods.

thy that titers of autoantibodies to both MDA-LDL and acrolein-LDL were highest in two subjects with clinical manifestations of atherosclerosis, whereas the lowest titer was found in a patient with cholesterol emboli syndrome, which occurred after aortic balloon counterpulsation therapy for shock. This suggests that intravascular dissemination of plaque material may have "absorbed" most of the circulating antibodies in this patient.

*Demonstration of oxidation epitopes on circulating LDL.* There is now evidence that minimally modified forms of LDL may exist in plasma (36, 56). To determine if any of the E0 series of antibodies would recognize epitopes on circulating LDL, we established a sensitive, double-layered sandwich chemiluminescent immunoassay. In this assay, goat anti–mouse IgG–$F_c$-specific antiserum (GAM-IgG) was coated on microtiter wells to which monoclonal antibody MB47 (57), which binds to apo B with high affinity, was then added as the bottom layers of the sandwich. (The GAM-IgG $F_c$ binds MB47 so as to maximize the expression of its two binding sites.) A 1:50 dilution of plasma was then added to allow binding of LDL, the middle of the sandwich. After extensive washing, an appropriate dilution of one of the IgM antibodies against OxLDL (the E0 antibodies) was then added as the top of the sandwich, and this in turn was detected by an alkaline phosphatase–labeled goat anti–IgM antibody. Fig. 9 displays a representative assay, showing mean values (±SD) of plasma from seven different subjects, for six different E0 antibodies, as well as non specific murine IgM (in place of E0) as a control. Antibodies E06 and E014 clearly recognized three to four times the amount of epitope on these LDL as did the other monoclonal antibodies or nonspecific IgM controls. Antibodies E03 and E07 also appeared to recognize epitopes on circulating LDL. We also demonstrated that this represents specific binding by appropriate competition studies; e.g., addition of OxLDL competed the ability of E014 to bind to the MB47-bound LDL under these conditions. In preliminary studies, there was considerable variation in the binding of E06 and E014 to LDL of different subjects, and studies to systematically test each of these monoclonals using a variety of plasma samples are currently under way. In separate experiments, we confirmed these findings by "inverting the sandwich." The E0 antibodies were plated, plasma added, and then MB47 (directly labeled with alkaline phosphatase) was used to document that LDL was bound by these antibodies. Again, E06 and E014 bound significantly more LDL than did the other E0 antibodies (data not shown). It is of particular interest that E06 is an antibody that recognizes an oxidized phospholipid epitope (32), while antibody E014 recognizes an MDA–lysine-like epitope, consistent with the data of Holvoet et al. (36). In separate and parallel wells, we used alkaline phosphatase–labeled MB24, another apo B–specific monoclonal, which recognizes a distinct and noncompeting epitope on apo B (58) to verify that equal amounts of LDL were bound by MB47 from each of the plasmas added; e.g., that under the assay conditions used, the amount of plasma LDL added saturates the capacity of MB47 binding.

## Discussion

It is likely that a large number of immunogenic structures are formed during the oxidation of LDL. When fatty acids undergo lipid peroxidation, a variety of highly reactive breakdown products are generated, such as MDA and 4-HNE, which in turn can modify lysine residues of apo B. The products formed

*Figure 7.* Photomicrographs showing the left shoulder area of an atheroma in a human brain artery. Serial sections were immunostained with natural and previously generated monoclonal antibodies as described in Fig. 6. (*A*), E04; (*B*), E06, (*C*), E011; (*D*), E014, (*E*), E017; (*F*), MDA-2, the induced monoclonal antibody to MDA-lysine; (*G*), control section stained without primary antibody (phase contrast); (*H*), HHF 35, an induced monoclonal antibody specific for muscle cell actin; (*I*), HAM 56, an induced monoclonal antibody to an epitope of human macrophages. (×139.)

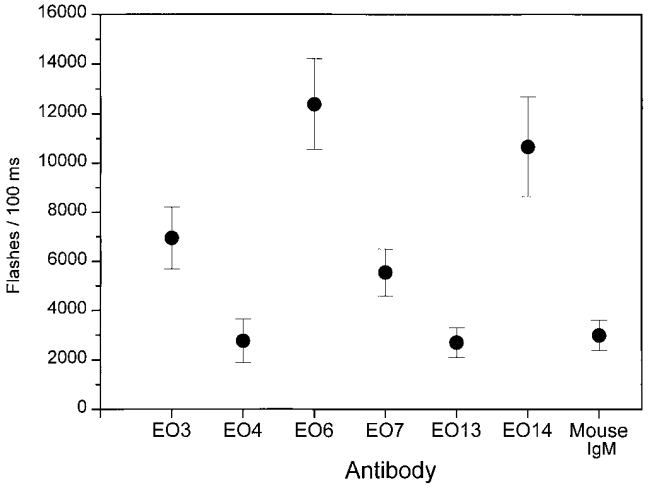


*Figure 9.* Detection of epitopes of OxLDL on circulating LDL by E0 antibodies. GAM-IgG $F_c$ was plated in 96-well microtiter plates, followed by monoclonal antibody MB47, specific for apo B. 50 μl of a 1:50 dilution of human plasma were then added. After 1 h of incubation, the wells were extensively washed. For each of the purified E0 antibodies, 50 μl (containing 10 μg/ml of the antibody) was then added and incubated for 1 h. After extensive washing with an automatic plate washer, the amount of E0 antibody bound (IgM) was detected by a goat anti–mouse IgM antibody labeled with alkaline phosphatase and chemiluminescent substrate. As a control, non specific mouse IgM was used in place of the E0 antibodies. Data shown are mean±standard deviation of plasma of seven different subjects. Results are reported as flashes of light/100 ms.

from even these simple aldehydes are highly complicated and many different structures are generated (28–30, 59). Oxidatively modified sterols (60, 61) and phospholipids (30, 31) are also generated when LDL is oxidized. Significant plasma titers of IgG and IgM autoantibodies against epitopes of OxLDL, in particular against MDA-LDL and 4-HNE-LDL, occur in a variety of experimental animals (6, 10). In preliminary studies in humans, the titers of autoantibodies to these epitopes were higher in subjects with increased cardiovascular disease or clinical manifestations of atherosclerosis (11–20). Furthermore, in LDL receptor-negative mice fed a high cholesterol diet, we found a fourfold rise in autoantibody titers to MDA-LDL, and the titer correlated with the extent of atherosclerosis (23).

Because autoantibody titers in apo E–deficient mice were unusually high (10) and approached those seen in mice after exogenous immunization, we thought that it would be possible to generate hybridomas from B cells isolated from their spleens, even in the absence of exogenous immunization (successful cloning of hybridoma cell lines depends on the presence of proliferating B cells). Indeed, from a fusion of B cells from the spleens of two apo E–deficient mice, we obtained 1,576 viable hybridomas. From these, between 32 and 64% secreted antibodies recognizing one or more epitopes of OxLDL, demonstrating an extraordinarily active immune response to this complex antigen. This point is further emphasized by the observation that the spleens of these mice were ~ 40% larger than those of age-matched C57BL/6 mice. Despite the fact that the plasma of apo E–deficient mice contained high titers of both IgG and IgM autoantibodies, we only cloned IgM monoclonal antibodies. The reasons for this are unclear.

Our data are consistent with the growing body of evidence that oxidation of LDL renders it very immunogenic. Indeed, Stemme et al. recently reported that 10% of a library of $CD4^+$ T-cells cloned from human carotid atherosclerotic plaques responded to OxLDL in an HLA-DR–specific manner (62), and Huang et al. just reported that even peripheral monocytes from normal individuals responded to OxLDL in an HLA class-II–specific manner (63). Because of the marked hyperlipidemia and the extensive atherosclerosis that occurs in apo E–deficient mice, there is undoubtedly a strong antigenic stimulus that is reflected in the vigorous immune response. The fact that we observed a high titer of IgM (Fig. 8), as well as IgG autoantibodies, to a variety of epitopes of OxLDL is consistent with an ongoing antigenic challenge produced by the continual generation of OxLDL (and/or other similar oxidatively modified structures). Evidence is also accumulating that activation of the immune system may modulate the atherogenic process and reduce lesion formation under certain conditions. For example, elimination of T lymphocytes with monoclonal antibodies accelerated atherosclerosis in balloon-catheterized rat aortas (64), and cyclosporine treatment increased atherosclerosis of hypercholesterolemic rabbits (65). Enhanced atherogenesis has also been described in some immune-compromised murine strains (66). Finally, we recently demonstrated that hyperimmunization of Watanabe Heritable Hyperlipidemic (WHHL) rabbits with homologous MDA-LDL induced very high titers of antibodies with similar specificity as naturally occurring autoantibodies, and that this intervention significantly reduced the progression of atherogenesis (25). If these results can be reproduced in other models of atherogenesis, this may indicate a new therapeutic approach, which would be greatly enhanced by a better understanding of the epitopes of OxLDL formed in vivo. For example, in the studies in WHHL rabbits, we used homologous MDA-LDL as immunogen because of the occurrence of natural autoantibodies with similar specificity. However, for future intervention studies, it would be helpful to know the specificity of the epitopes of OxLDL that trigger a strong immune response in vivo, to generate modified LDL or even synthetic immunogens that contain these epitopes. The availability of the natural monoclonal antibodies described here should assist in this endeavor.

In our initial screening strategy, we used several models of OxLDL; MDA-LDL, 4-HNE-LDL, as well as $Cu^{2+}$-LDL. Although we initially also identified monoclonal antibodies that appeared to bind exclusively to 4-HNE-LDL, these were lost during the cloning procedure. A characterization of the 13 monoclonal antibodies finally selected indicated that as a group they recognized epitopes of $Cu^{2+}$-LDL or MDA-LDL. When LDL was subjected to increasing degrees of oxidation, each of these 13 monoclonal antibodies recognized epitopes in the OxLDL preparation, but there was considerable variation in the extent of recognition over time, and in particular, with each LDL preparation. This was most notable in the native LDL preparation; e.g., LDL plated at zero time. It is unclear if this represents binding to epitopes generated ex vivo, after the LDL was isolated, or if this represents the sensitive detection of epitopes that existed on LDL even in the circulation. As shown in the experiment depicted in Fig. 9, there is indeed binding of several of these antibodies to epitopes on circulating LDL. In this experiment, LDL was directly captured from plasma into the microtiter well by use of the apo B–specific monoclonal antibody, MB47, and was always protected by

plasma or EDTA-containing buffers, thus minimizing any possibility of ex vivo oxidation. Other experiments, in which the sandwich assay was inverted and several of the E0 antibodies were used to capture material from plasma, again showed that E06 and E014, in particular, captured LDL from plasma. Thus, some circulating LDL particles do contain selected oxidation-specific epitopes, consistent with several recent reports (36, 56, 67, 68). The origin of these minimally modified LDL particles (reviewed in reference 4) is not clear. Because of the extensive antioxidant defenses of plasma, it seems unlikely that oxidation would occur in the circulation. However, LDL could have been modified elsewhere in the body (e.g., in the liver, reference 25), or during an earlier passage through the arterial wall.

We have previously generated antisera and monoclonal antibodies to MDA-lysine, 4-HNE-lysine and against epitopes of $Cu^{2+}$-LDL (6, 7). The fact that many of the E0 monoclonal antibodies bind to these two model epitopes of OxLDL, as well as to $Cu^{2+}$-LDL, and the fact that the E0 antibodies immunostain atherosclerotic lesions of rabbit and man, strongly supports the presence of these epitopes of OxLDL in vivo. As noted above, a large number of different epitopes are likely to be generated during the oxidation of LDL. In the present study, we have demonstrated that there are autoantibodies to acrolein-modified LDL, as well as to epitopes on LDL generated by condensation of AOP-LDL and LOP-LDL. This suggests that autoantibodies are directed at a large number of different immunogenic structures generated by the oxidation of LDL. Furthermore, the fact that many of the E0 antibodies recognize not only modified LDL, but other similarly derivatized proteins (e.g., E014 recognizes not only MDA-LDL but also MDA-HDL and MDA-BSA) suggests that once an antibody is generated against an adduct between a lipid oxidation product and the apoprotein, it could also react with similar adducts formed elsewhere in the body as a result of a proinflammatory setting. For example, oxidation of LDL (or HDL) also leads to the generation of modified phospholipids (30, 32, 67, 68) that appear to be immunogenic. In the companion paper (32), we demonstrate that several of the E0 antibodies selected for binding to OxLDL (e.g., E06) bind to epitopes of oxidized phospholipids that contain polyunsaturated fatty acids, such as cardiolipin or phosphatidylserine. In a similar manner, antibodies recognizing OxLDL are likely to react with many other structures. For example, MDA2, directed against MDA-lysine, has been shown to immunostain iron-bearing hepatocytes of iron-overloaded rats (69), neurons in patients with Alzheimer's disease (70) and multiple sclerosis (71), lungs of rats perfused with advanced glycation endproduct–complexes, which initiate oxidative stress in endothelial cells (72), and the glomerulus of rats with autoimmune glomerulonephritis (73). In fact, it is possible that many of the autoantibodies to epitopes of OxLDL represent anamnestic responses to similar epitopes generated elsewhere (and vice versa).

We and others have previously generated antibodies to OxLDL by immunizing mice with predicted models of OxLDL; e.g., murine LDL modified in vitro with MDA (6, 7, 33). A priori, one cannot predict whether such model epitopes actually occur in vivo. However, the cloning of natural autoantibodies as described here should yield antibodies that are specifically directed against epitopes formed in vivo, and should be valuable in purifying and defining specific epitopes. In turn, such defined chemical structures would be extremely useful in developing highly standardized assays to detect the presence of autoantibodies in plasma, an issue of potential clinical relevance, since the titer of such autoantibodies appears promising as an indicator of the extent of atherosclerosis (74). Currently, the reproducible generation of antigens for such assays; e.g., OxLDL, or even MDA-LDL, constitutes a major problem. In addition, the availability of well defined natural epitopes of OxLDL could be very useful in future studies designed to increase the impact of immunization on the atherogenic process. Furthermore, several of the E0 antibodies appear to recognize epitopes of OxLDL on circulating LDL. It will be important to determine if the extent of expression of these epitopes on plasma LDL reflects the degree of ongoing in vivo oxidation and/or the extent of atherosclerosis, and if the level of epitope expression differs in patients with clinical manifestations of atherosclerosis. Finally, monoclonal antibodies binding preferentially to certain sites of lesions may potentially be useful for diagnostic purposes.

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