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Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis

(modified lipoproteins/oxidation/autoantibodies/atherosclerosis/immune system)

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ABSTRACT **Atherosclerotic lesions contain oxidized LDL (OxLDL), immunoglobulins, and immune-competent cells. Low levels of circulating autoantibodies against malondialdehyde (MDA)-modified lysine, an epitope of OxLDL, occur in several species, and immune complexes between such autoantibodies and OxLDL are present in lesions. To study the potential role of autoantibodies against OxLDL in the atherogenic process, we prospectively hyperimmunized LDL receptor-deficient rabbits with homologous MDA-LDL and determined the effects of this intervention on the development of atherosclerosis. Immunization with MDA-LDL generated high titers of antibodies with similar specificity as naturally occurring autoantibodies. Immunized animals showed a significant reduction in the extent of atherosclerotic lesions in the aortic tree after 6.5 months, compared with "saline"-immunized controls (48% vs. 68%, $P < 0.005$). Immunization with keyhole limpet hemocyanin produced no change in lesion formation. Although the mechanisms by which immunization led to a protective effect are unknown, these results suggest an important role for the immune system in modulating the atherogenic process and may indicate a novel approach for inhibiting the progression of atherosclerosis.**

Substantial evidence indicates that oxidized LDL (OxLDL) contributes to atherogenesis by a number of mechanisms (1, 2). Furthermore, even minor modifications of LDL render it highly immunogenic (3), and circulating autoantibodies recognizing several forms of OxLDL, in particular malondialdehyde (MDA)-modified lysine, are prevalent in humans and other species (4–6). These autoantibodies are capable of binding to epitopes of OxLDL in lesions (4, 5, 7), and immunoglobulins isolated from lesions of Watanabe heritable hyperlipidemic (WHHL) rabbits and humans recognize OxLDL and are present in lesions, in part as immune complexes with OxLDL (8).

It is currently unknown if the titer of autoantibodies against epitopes of OxLDL is merely an indicator of lipoprotein modification or if such autoantibodies could modulate the atherogenic process. To address this question, we hyperimmunized WHHL rabbits with homologous MDA-LDL and determined the effect of this intervention on atherosclerosis.

METHODS

Immunization and Antibody Determination. LDL was isolated from healthy WHHL donor rabbits by sequential ultracentrifugation in the presence of antioxidants and antiproteolytic agents and was modified with MDA (9). MDA-LDL in which 70–85% of the lysine residues were modified was used as the immunogen and for the determination of antibody titers (9). Immunization of WHHL rabbits was begun either at an age of 6 weeks or 6 months. The primary immunization consisted of a subcutaneous injection of 160 μg of MDA-LDL (protein) per kg of body weight dissolved in phosphate-buffered saline (PBS) and suspended in an equal volume of Freund's complete adjuvant. Booster immunizations consisted of antigen in Freund's incomplete adjuvant injected intramuscularly 3 and 5 weeks after the primary immunization and subsequently at monthly intervals for 6 months. Control groups were immunized with sterile PBS or keyhole limpet hemocyanin (KLH) (Sigma) prepared with Freund's complete or incomplete adjuvant in a similar way. Serum aliquots were frozen at −20°C, and autoantibody titers against MDA-LDL were determined by solid-phase RIA in a single assay at the end of the experiment with human MDA-LDL as antigen (9). Antibody titers were defined as the reciprocal of the greatest serum dilution that showed specific binding 3 times greater than the preimmunization serum (9).

Morphometric Determination of Atherosclerosis. After 6.5 months, rabbits were sacrificed by pentobarbital overdose. The aortic tree was perfusion-fixed and dissected from the aortic valve to the iliac bifurcation (5, 7). Morphometric measurements were performed as described (5), and results were expressed as the percent of the thoracic, abdominal, or entire aorta covered by atherosclerotic lesions. Data were compared by using Student's unpaired t test.

Immunocytochemistry. After image analysis, six cross sections of ≈5 mm each were taken from defined anatomical locations of the aorta: (*i*) ascending arch, (*ii*) 1 cm distal from section *i*, (*iii* and *iv*) second and fifth intercostal artery orifices, respectively, (*v*) abdominal aorta 1 cm distal from the diaphragm, and (*vi*) 5 cm distal from location *v*. Tissues were immunostained with an avidin–biotin–alkaline phosphatase system (Vector Laboratories) (5, 7) with the following monoclonal antibodies: MDA2, specific for MDA-lysine (9); NA59, specific for 4-hydroxynonenal (4-HNE)-modified lysine (9); RAM 11, specific for macrophages (a gift from Allen Gown, University of Washington); MW6-B, a purified ascites against rabbit major histocompatability complex (MHC) class II; MM4-B, a purified ascites against rabbit CD25 [interleukin 2 (IL-2) receptor α chain] (10) (Spring Valley Laboratories, Sykesville, MD); L11/135, a pan-T-lymphocyte antibody (purified ascites) [a gift of Peter Libby (Harvard Medical School) and Göran Hansson (Gothenburg University)] (11); and HHF-35, specific for muscle cell actin (Enzo Diagnostics). Primary antibodies bound to the tissue were detected with appropriate biotinylated anti-mouse immunoglobulin sera (Vector Laboratories). Endogenous rabbit IgG and IgM present in lesions were detected by using biotinylated antibodies against rabbit

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Abbreviations: LDL, low density lipoprotein; OxLDL, oxidized LDL; MDA, malondialdehyde; WHHL, Watanabe heritable hyperlipidemic; KLH, keyhole limpet hemocyanin; IL-2, interleukin 2; MHC, major histocompatibility complex; 4-HNE, 4-hydroxynonenal.

IgG and IgM, respectively (Vector Laboratories). Complement component C3 was immunostained with goat anti-rabbit C3 antiserum (Organon Teknika–Cappel). Control slides were incubated without primary antibody.

RESULTS

Preliminary Experiment. As neither the impact of age on the immune response of rabbits nor the stage of atherosclerotic lesions potentially influenced by immune processes were known *a priori*, a preliminary experiment was carried out with two groups of nine WHHL rabbits each. Immunization was initiated in one group at 6 weeks of age and in the second group at 6 months. Three animals in each group were immunized with homologous MDA-LDL, three with KLH (to obtain KLH-specific antibodies, which should not bind to any endogenous antigen), and three with PBS. RIAs confirmed that all animals immunized with MDA-LDL developed high titers of antibodies against either rabbit or human MDA-LDL (titers > 100,000; $n = 6$). All animals immunized with KLH responded with high titers of antibodies against KLH (titers > 100,000; $n = 6$). In contrast to previous studies using older WHHL rabbits (4), the titers of autoantibodies to MDA-LDL in the PBS- and KLH-immunized groups initially were very low (e.g., titers ≤ 100 at 6 weeks of age) but increased with age. Body weights, plasma cholesterol, and triglyceride levels did not differ significantly between groups. After 6.5 months, the extent of aortic atherosclerosis was determined. As expected, the older groups showed a much higher percentage of lesions in the entire aorta than the groups started shortly after weaning (71% vs. 26% in the respective PBS groups). In the older groups, animals with high titers of antibodies to MDA-lysine generally had fewer lesions than animals in the KLH and PBS groups. Despite the small number of animals in each group, the difference between the MDA-LDL group and the PBS group was significant in the thoracic segment including the arch (65.0% ± 2.5% vs. 84.6% ± 5.6%, $P < 0.05$) and in the entire aorta (58.1% ± 4.5% vs. 71.5% ± 5.2%, $P < 0.05$). The extent of atherosclerosis in the KLH and the PBS groups was not significantly different from each other. By contrast, in animals in which immunization had been started at 6 weeks of age, no significant differences were seen between MDA-immunized and KLH and PBS control animals (27.4% ± 6.0% in the MDA-LDL group, 26.3% ± 1.1% in the PBS group, and 27.4% ± 2.9% in the KLH group; not significant).

Main Experiment. Based on these data, immunization of animals in the main experiment was started at 6 months of age. Two litter-matched groups of 11 rabbits each (male and female) were immunized with MDA-LDL or PBS. After several months, 3 animals of the control group died from severe diarrhea, so that the total number of animals studied was 11 in the MDA-LDL group and 8 in the PBS group. As before, all MDA-LDL-immunized animals developed high titers of IgG-type antibodies recognizing MDA-LDL (Fig. 1). Titers were >65,000 in 1, >100,000 in 7, and >250,000 in 3 animals. Autoantibody titers in the PBS group were in the same range as were found in the sera prior to immunization in the MDA-LDL group (>1,024 to >4,048). Titers of IgG autoantibodies to MDA-LDL in the PBS group remained low and relatively constant throughout the experiment (Fig. 1 *Inset*). By contrast, titers in the MDA-LDL-immunized group quickly reached a plateau and remained fairly constant thereafter.

Previously, we expressed the titers of autoantibodies to MDA-LDL as the ratio of binding of IgG to MDA-LDL divided by the binding to native LDL (12). Such a ratio corrects for nonspecific binding to native LDL and indicates the specificity of the antibodies for modified lysine epitopes. At the end of the 6.5 months of intervention ("T4 time point"), the binding ratio of IgG- and IgA-type antibodies was higher in MDA-LDL-immunized animals than in controls (Fig. 2). The differences were significant to a serum dilution of 1:100,000 for IgG- and to 1:1024 for IgA-type

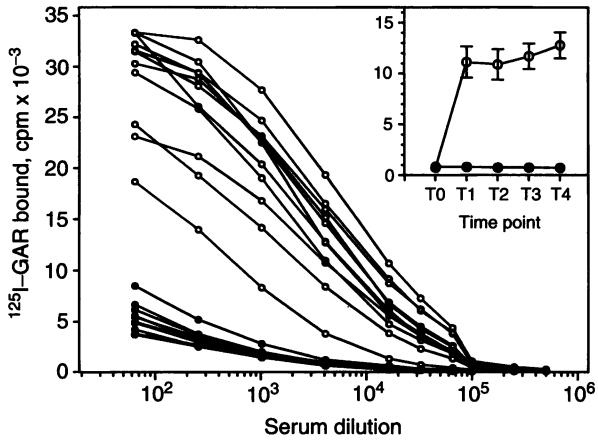

FIG. 1. Dilution curves of autoantibodies to human MDA-LDL in the serum of rabbits immunized with rabbit MDA-LDL. The antigen was plated at 5 μg/ml, and the amount of antibody bound was detected with ^{125}I-labeled goat anti-rabbit immunoglobulin (^{125}I-GAR). Binding of sera before immunization (●) and of sera obtained after 6.5 months (○) is shown for all MDA-LDL-immunized rabbits of the main experiment ($n = 11$). (*Inset*) Binding of antibodies in the sera of the MDA-LDL immunized (○) and the PBS control group (●) to MDA-LDL over time. The figure represents binding at a single serum dilution (1:4096) prior to immunization ("T0" time point) and at approximately 1.5, 2.5, 5, and 6.5 months after the primary immunization ("T1–T4" time points). Values are means ± SEM ($n = 11$ in the MDA-LDL group and $n = 8$ in the PBS group).

antibodies ($P < 0.05$ to $P < 0.0001$). By contrast, no differences between the two groups were found in the titers of IgM-type antibodies. The time course of development of IgA antibodies in the MDA-LDL-immunized group resembled that of IgG. In contrast, there were no significant changes over time in the control group. No significant changes in the titer of IgM antibodies were observed over time in either the control or immunized group.

Sera drawn from several rabbits after 6.5 months of immunization with MDA-LDL were used in competitive RIAs to determine the specificity of the high-titered antibodies in these sera. As shown in Fig. 3, the binding of serum antibodies to the plated antigen (human MDA-LDL) was almost completely blocked by competition with MDA-LDL. Although other MDA-lysine-containing proteins, [e.g., MDA-albumin and MDA-polylysine (M_r, 4000)] also showed significant competition, the competition curves were not parallel, suggesting that the relative affinity of the

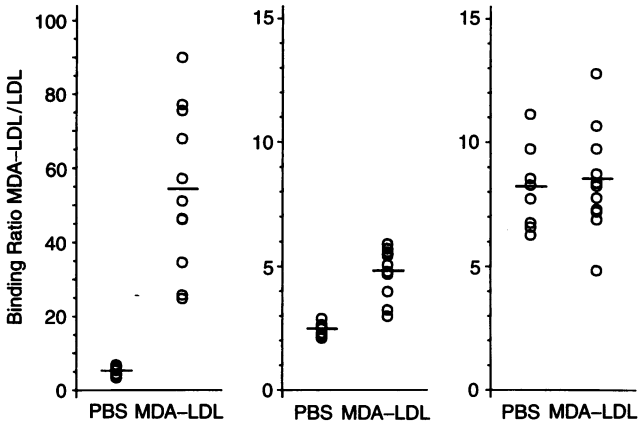

FIG. 2. Autoantibodies of the IgG (*Left*), IgA (*Center*), and IgM (*Right*) type in the sera of MDA-LDL-immunized and control animals after 6.5 months of immunization. Data are presented as the ratio of the antibody binding to MDA-LDL divided by the binding to native LDL. Serum dilution was 1:4096 for IgG-type, 1:1024 for IgM-type and 1:64 for IgA-type antibodies. Differences were significant at $P <$ 0.0001 for IgG and IgA but not significant for IgM.

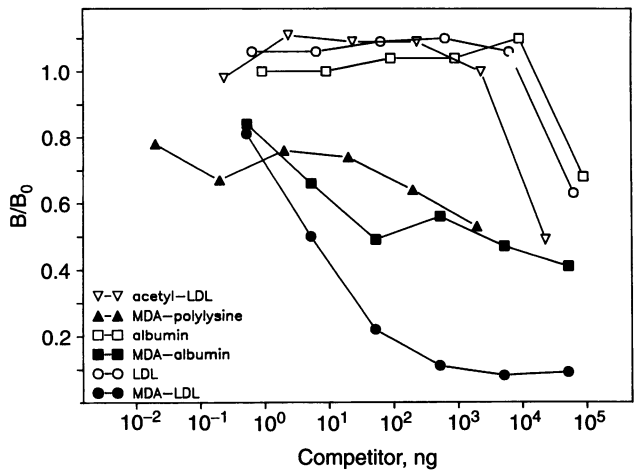

FIG. 3. Competitive solid-phase RIA demonstrating the MDA-lysine specificity of antibodies in the serum of an MDA-LDL-immunized rabbit obtained at the T4 time point (see Fig. 1). Human MDA-LDL was plated as antigen at 1 μg/ml, and 25 μl of a 1:5000 dilution of plasma was added together with an equal volume of PBS containing increasing concentrations of competitors. Results are reported as the ratio of binding in the presence of competitor (B) divided by the binding in the absence of competitor (B_0).

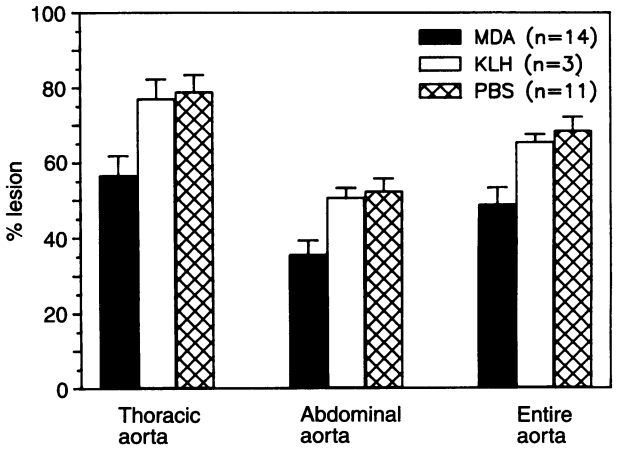

FIG. 4. Percent of the aortic surface covered by atherosclerotic lesions. Data represent all rabbits whose immunization was begun at age 6 months, including the ones from the preliminary experiment. Lesion areas in the MDA-LDL-immunized group was less than in the PBS or KLH groups in the entire aorta ($P < 0.005$) and in the thoracic and abdominal segments ($P < 0.005$). Differences between the PBS and KLH group were not significant. Values are means ± SEM.

antibodies was greater for MDA-LDL than that for other competitors. The competition with "native" LDL may be due to minor degrees of oxidation that may have occurred *ex vivo* despite our attempts to prevent this (9). Thus, the specificity of the antibodies induced by continuous boosting with MDA-LDL over 6.5 months resembled that of "genuine" autoantibodies present in rabbits and humans (4).

The immunized and control groups showed no significant differences in initial body weights or the rate of weight gain throughout the 6.5 months of the experiment. The plasma cholesterol levels also were similar in both groups (time averaged total cholesterol = 531 ± 37 mg/dl in the immunized group and 608 ± 76 mg/dl in the control group, not significant). Since body weights and cholesterol values were similar in the rabbits of the preliminary experiment, data from the 19 animals of the main experiment were combined with those from the 9 animals of the preliminary experiment in which immunization was also begun at 6 months of age.

Compared with controls, the extent of atherosclerosis was markedly reduced in the MDA-LDL-immunized group (Fig. 4). This was true in the entire aorta (48.80% vs. 68.47%, $P < 0.005$) as well as in individual segments (arch and thoracic aorta: 56.63% vs. 78.72%, $P < 0.005$; abdominal aorta: 35.57% vs. 52.35%, $P < 0.005$).

To determine whether the reduction in atherogenesis was reflected in altered lesion morphology, we utilized immunocytochemistry for a qualitative assessment of immune-competent cells, immunoglobulins, or oxidation-specific epitopes (Fig. 5). This was performed on six cross sections of defined areas of the aortic tree, using sections from the two immunized animals with the most and two animals with the least atherosclerosis as well as from two saline controls with the most atherosclerosis. All sections studied showed the presence of epitopes of OxLDL, such as MDA-lysine (Fig. 5*H*) and 4-HNE-lysine (not shown), in a distribution typical of the respective stage of lesion. Immunoglobulins M and G (Fig. 5 *E* and *F*) and complement C3 (not shown) were also prevalent in most lesions. Lesions contained large numbers of macrophage/foam cells (Fig. 5*G*) as well as a much smaller number of T cells (Fig. 5*D*). T lymphocytes were seen in lesions ranging from very early fatty streaks to very advanced plaques. In more advanced lesions, T cells were predominantly found in the superficial layers of the intima. Isolated T cells in the deeper portions of the lesion (as shown in Fig. 5) occurred less frequently. Expression of MHC class II antigen was prevalent in areas containing immune-competent cells (Fig. 5*B*). Staining of lesion areas with a monoclonal antibody to rabbit CD25 (IL-2 receptor) indicated that many of the intimal leukocytes (both T cells and mostly macrophages) were activated (Fig. 5*C*). Nonlesioned areas of the aorta did not stain with this antibody. However, when sections from MDA-LDL immunized and control animals were compared, no gross differences were seen in the staining patterns obtained with any of these antibodies.

DISCUSSION

In this study, greatly exaggerated "autoantibody" titers to MDA-lysine, an epitope of OxLDL, were induced in WHHL rabbits. After 6.5 months, rabbits with high titers showed significantly less atherosclerosis throughout the aorta than litter-matched controls immunized with PBS or KLH, which had much lower titers of natural autoantibodies to MDA-lysine.

The involvement of immune mechanisms in atherogenesis is increasingly recognized (reviewed in ref. 13). Human atherosclerotic lesions contain large numbers of immune-competent cells, predominantly macrophages, as well as $CD4^+$ and $CD8^+$ T lymphocytes, and several observations indicate that immune-competent cells in the intima are activated. A significant percentage of intimal T lymphocytes are $CD25^+$ (i.e., express IL-2 receptors), and many monocyte/macrophages also seem to express IL-2 receptors (Fig. 5*C*) (14). [It is also possible that some of the staining noted in Fig. 5*C* represents the extracellular form of IL-2 receptors, which is known to be released by activated cells, as in states of autoimmunity (15).] MHC II antigens are expressed both on endothelial cells and on vascular smooth muscle cells in the vicinity of T lymphocytes (16), presumably as a result of γ interferon secreted by activated T cells. The presence of large amounts of immunoglobulins and complement is also well known, and immune complexes consisting of OxLDL and autoantibodies to epitopes of OxLDL are also found in lesions (8). Finally, the presence of terminal C5b-9 complement complexes and the expression of complement receptors by vascular cells is well documented (17, 18).

The mechanism by which immunization with homologous MDA-LDL reduced atherosclerosis in our study is unknown, but several pathways could have contributed to the protective

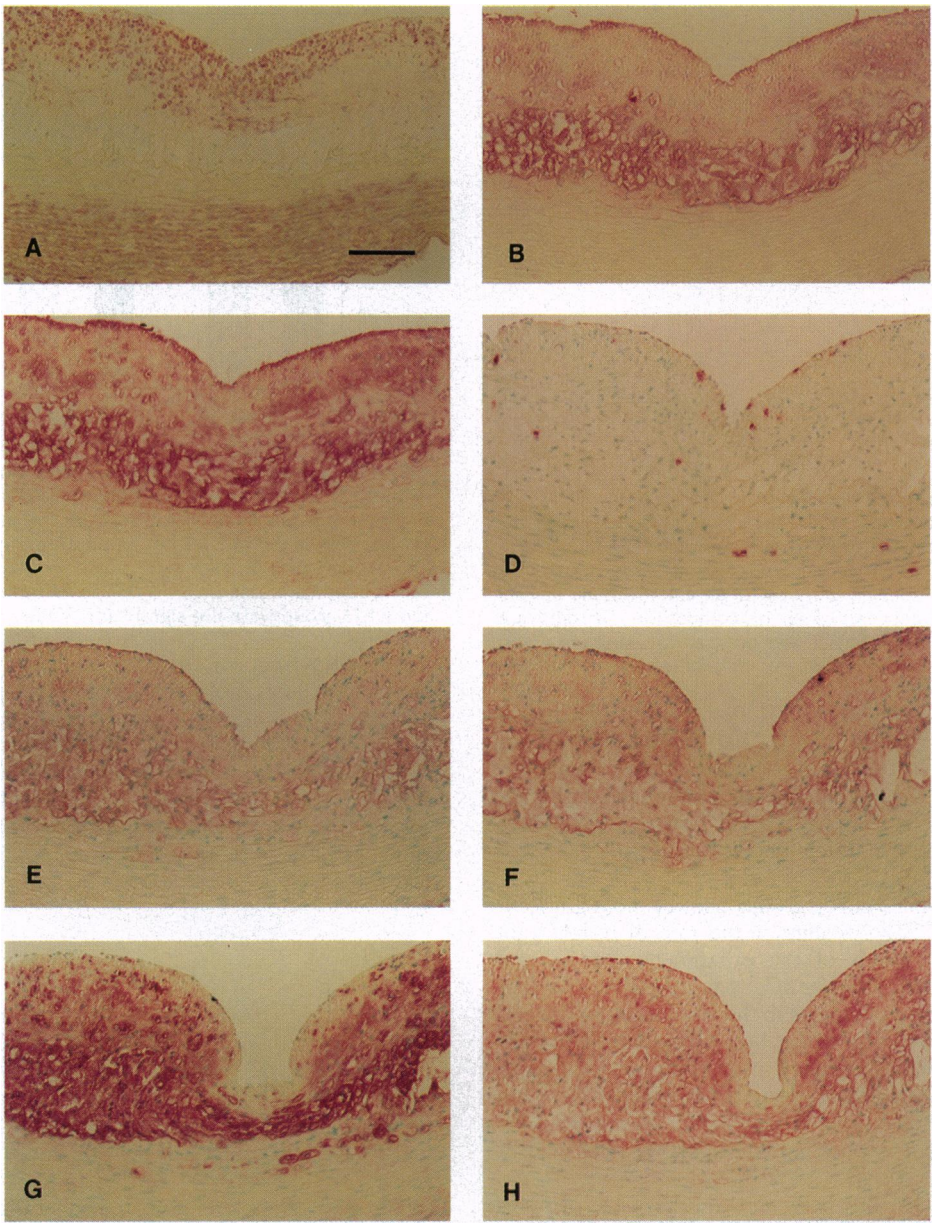

FIG. 5. Immunostaining of serial sections of the thoracic aorta of a saline-immunized WHHL rabbit. Epitopes recognized are indicated by a red color; the nuclei are counterstained with methyl green. (*A*) HHF-35 (dilution 1:3000), specific for muscle cell actin. Staining indicates that many of the intimal cells in this advanced lesion are of smooth muscle cell origin. (*B*) MW6-B, specific for rabbit MHC class II (dilution 1:100). (*C*) MM4-B, specific for CD25 (IL-2 receptor) (dilution 1:100). Staining indicates activation of intimal leukocytes (predominantly macrophages). (*D*) L11/135 (dilution 1:25), specific for T cells. (*E* and *F*) Polyvalent antisera against rabbit IgM and IgG (1:200). (*G*) RAM 11 (dilution 1:1000), specific for macrophages and macrophage-derived foam cells. (*H*) MDA2 (dilution 1:400), specific for MDA-lysine, an epitope of OxLDL. Similar patterns were obtained with NA59, specific for 4-HNE-lysine epitopes. (×83; Bar = 100 μm.)

effect. First, the antibodies could have eliminated from the circulation LDL that had undergone minimal degrees of oxidative modification. Indeed, recent evidence indicates that some LDL particles in plasma are modified (19). Such modifications of LDL do not necessarily have to occur in plasma, where excellent antioxidant defenses exist, but could occur in LDL particles passing through the artery wall or other tissue sites, particularly in a state of enhanced oxidative stress induced by marked hypercholesterolemia (20, 21). Thus, MDA-lysine might occur on some LDL particles, and high titers of circulating autoantibodies might promote their rapid elimination, preventing them from reentering the arterial wall. We previously demonstrated such rapid removal of minimally glycated LDL injected into rabbits immunized with homologous glycated LDL (22).

High titers of antibodies to OxLDL may also promote more rapid removal of OxLDL from the intima by enhancing the macrophage uptake of immune complexes containing OxLDL via the Fc receptor pathway (23, 24). *A priori*, one would assume that this would enhance foam cell formation, and thus increase lesion formation. However, it is now apparent that both early and late products of LDL oxidation have many proatherogenic effects (2, 25). A more efficient clearance of OxLDL (along with its complement of proatherogenic components) from the intima may therefore be biologically desirable (26), even though this occurs at the expense of intracellular accumulation of cholesteryl esters.

A third mechanism potentially contributing to the beneficial effect could be provided by enhancement of cell-mediated immunity, such as that caused by increased numbers of im-

mune competent cells in lesions or by their increased activation. OxLDL is chemotactic for both monocytes and T cells (26) and may activate T cells in the presence of monocytes (27). Most importantly, Stemme *et al.* (28) have separated and cloned $CD4^+$ cells from human atherosclerotic plaques and demonstrated that up to 10% of such clones are specifically stimulated by OxLDL to proliferate in an HLA-dependent manner. The presence of immune complexes of OxLDL may serve to activate T cells even more effectively (29). Evidence for a beneficial role of activated T cells was provided by Hansson *et al.* (30), who demonstrated that the elimination of T lymphocytes with monoclonal antibodies resulted in larger proliferative lesions in balloon-catheterized rat aortas. Furthermore, cyclosporine A-treated mice exhibited increased atherosclerosis, which may be due in part to the depletion of suppressor T cells (31). Finally, Fyfe *et al.* (32) recently reported that class I MHC-deficient C57BL/6 mice (which lack cytolytic T cells and have impaired natural killer cell activity) develop a 3-fold increase in lesions in the aortic valve region when fed a high-fat diet. Thus, the immunization may have led to both humoral and cell-mediated immune response. Whatever the mechanisms involved, our studies are consistent with an important role of the immune system in atherogenesis and suggest a novel approach for inhibiting its progression.

In a few preliminary studies in humans, higher autoantibody titers to epitopes of OxLDL have been correlated with increased atherosclerosis (12, 33, 34). This suggests that autoantibodies may be an indicator of the extent of atherosclerosis. It is noteworthy that transgenic mice lacking apoprotein E, characterized by marked hypercholesterolemia and spontaneous atherosclerosis, have very high autoantibody titers to MDA-LDL (5) and that an elevated autoantibody titer in LDL-receptor negative mice fed a cholesterol-enriched diet correlates with the extent of atherosclerosis (W.P., R. K. Tangirala, E.M., and J.L.W., unpublished data). Whether the immune response exerts a beneficial role in these mice (e.g., whether atherosclerosis would be worse in the absence of an immune response) is unknown. The availability of murine strains with various immune defects should make it possible to determine the role of the immune system in atherogenesis.

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