

Autoantibodies to OxLDL fail to alter the clearance of injected OxLDL in apolipoprotein E-deficient mice

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Abstract This study tests the hypothesis that autoantibodies to oxidation epitopes on oxidized LDL (OxLDL) promote the clearance of OxLDL from the plasma. Human LDL (hLDL) was injected into immune-competent apolipoprotein E-deficient (apoE^{-/-}) mice and immune-deficient apoE^{-/-}/recombination-activating gene-deficient mice that lack mature T and B cells and thus antibodies. There was a progressive decrease in human apoB-100 in the plasma in all mice, but the rate of clearance was not greater in the immune-competent mice than in the immune-deficient mice. Interestingly, oxidized phospholipid (OxPL) epitopes as detected by the EO6 antibody on the hLDL increased over time, suggesting de novo oxidation of the LDL or transfer of OxPL to the particles. Because the native LDL was not extensively modified, we also examined the clearance of copper OxLDL. Although the extensively OxLDL was cleared faster than the native LDL, there was no difference in the rate of clearance as a function of immune status. There appeared to be some transfer of OxPL to the endogenous murine LDL. Together, these results suggest that oxidation-specific antibodies do not participate to any great extent in the clearance of OxLDL from plasma. However, it is possible that such antibodies may bind to oxidation epitopes and modulate lesion formation within the vessel wall.—Reardon, C. A., E. R. Miller, L. Blachowicz, J. Lukens, C. J. Binder, J. L. Witztum, and G. S. Getz. **Autoantibodies to OxLDL fail to alter the clearance of injected OxLDL in apolipoprotein E-deficient mice.** *J. Lipid Res.* 2004. 45: 1347–1354.

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It is now established that 65–75% of the clearance of native LDL in vivo is mediated by the LDL receptor pathway, primarily in the liver (1). The fact that macrophage foam cell formation and atherosclerosis are greatly accelerated in animals or human subjects with complete genetic defi-

ciency of the LDL receptor focused attention on alternative pathways and led to the recognition of scavenger receptors that bound modified LDL (2), such as oxidized LDL (OxLDL). Although the enhanced uptake of OxLDL by macrophages originally attracted attention to the oxidation of LDL as a major atherogenic factor, it is now appreciated that OxLDL and its many oxidized lipid moieties contribute to atherosclerosis by numerous proinflammatory mechanisms, such as the induction of chemotaxis of monocytes and T cells, and by the induction of proinflammatory and proatherogenic genes in vascular wall cells (3–5).

OxLDL is taken up by macrophages by a variety of scavenger receptors (6). Although these pathways represent a mechanism by which the formation of foam cells occurs, they also represent pathways mediating the clearance of OxLDL from the circulation. Oxidation of LDL results in the formation of a variety of oxidation-specific neoepitopes that lead to immune responses. In addition, atherogenesis in both experimental animals and humans is associated with increased concentrations of autoantibodies to epitopes of OxLDL (7–10). This is particularly the case in the commonly used murine model of atherosclerosis, the apolipoprotein E-deficient (apoE^{-/-}) mouse. The precise role of these autoantibodies in the pathogenesis of atherosclerosis is unknown for the most part. However, their involvement is strongly indicated by experiments in rabbits and mice involving immunization with OxLDL or malondialdehyde-modified LDL (MDA-LDL), which have demonstrated atheroprotection (11–15). Although the mechanism(s) by which such immunization provides atheroprotection is largely unknown, one likely mechanism would be the promotion of enhanced clearance of early forms of OxLDL from the circulation,

Abbreviations: apoE, apolipoprotein E; hLDL, human LDL; MDA-LDL, malondialdehyde-modified LDL; OxLDL, oxidized LDL; OxPL, oxidized phospholipid; PC, phosphorylcholine; RAG, recombination-activating gene 2; RLU, relative light units.

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thereby diverting such modified particles from the artery wall. Indeed, in previous studies in which nonenzymatically glycosylated LDL was injected into rabbits that had high antibody titers to glycosylated LDL, such enhanced plasma clearance was demonstrated (16). On the other hand, atherosclerosis proceeds in hypercholesterolemic mice, albeit to a reduced extent in some anatomical sites, even in the presence of complete immune deficiency (17–19), a situation in which antibody formation is absent.

Because of the robust autoimmune response to epitopes of OxLDL in apoE^{-/-} mice, it was possible to clone a panel of monoclonal autoantibodies from the spleens of these mice that were directed to epitopes of OxLDL (20). In particular, one immunodominant clonotypic set of IgM autoantibodies was identified, such as the prototypic antibody EO6, which bears the T15 idiotype and which was shown to specifically bind to the phosphorylcholine (PC) moiety of oxidized PC-containing phospholipids, such as those present in OxLDL (21). However, EO6 does not bind to the PC moiety of unoxidized phospholipids, as found in native LDL, even though they contain the same PC moiety. Antibodies, such as EO6, can be used to measure the presence in plasma of OxLDL bearing oxidized phospholipid (OxPL) epitopes. In this study, we have taken advantage of the complete immunodeficiency of apoE^{-/-} mice to learn whether naturally occurring autoantibodies to OxLDL epitopes play a role in the metabolism of circulating forms of OxLDL, for example, to promote enhanced clearance of OxLDL from the circulation. Minimally modified forms of OxLDL, which would not react with scavenger receptors, might form immune complexes with such antibodies, leading to enhanced clearance through Fc receptors on reticuloendothelial cells. If such a mechanism were a major contributor to the *in vivo* clearance of OxLDL, one would expect, first, that levels of OxLDL in plasma of immune-deficient animals would be increased compared with those of immune-competent controls, and second, that the plasma clearance of OxLDL would be delayed in the immune-deficient animals. Neither of these expectations was observed in this comparison of immune-deficient and immune-competent apoE^{-/-} mice.

EXPERIMENTAL PROCEDURES

Preparation of native and copper OxLDL

LDL ($d = 1.019\text{--}1.062$ g/ml) was isolated from normolipemic human plasma by sequential ultracentrifugation (22). The plasma was adjusted to 0.27 mM EDTA, pH 7.4, 2 mM benzamidine, 1 μ M D-Phe-Pro-Arg chloromethylketone, 0.01% aprotinin, 50 μ g/ml chloramphenicol, 100 μ g/ml gentamycin, and 1 mM PMSF immediately after collection. After isolation, the LDL was dialyzed against PBS. MDA-LDL and copper OxLDL were prepared as previously described (9) with minor modifications. To prepare OxLDL, the isolated LDL was extensively dialyzed against PBS buffer to remove the EDTA before modification. It was adjusted to 1 mg of LDL per 1 ml of PBS and incubated with 10 μ M copper sulfate for 16 h. After these incubations, the EDTA was added back and the OxLDL preparations were dia-

lyzed against PBS containing 0.27 mM EDTA to remove the copper sulfate. All samples were sterilized by filtration before determining protein concentration (23) and stored at 4°C until use.

Injection of LDL into mice

Female apoE^{-/-} mice and apoE^{-/-}RAG2^{-/-} mice (deficient in both apoE and recombination-activating gene 2) (18) (all mice were backcrossed to C57BL/6 mice for three or four generations) were maintained either on chow diet (Harlan TekLab) or a diet of 0.25% cholesterol and 18% milk fat (Harlan TekLab; TD97222) for 8 weeks starting at 8 weeks of age. The high-fat diet was fed because this may enhance the difference in the rate of clearance of the injected human LDL (hLDL) between the immune-competent and immune-incompetent mice. Plasma cholesterol levels were determined from blood samples obtained after 1 month on each diet via enzymatic assays using a kit supplied by Roche Diagnostics, Inc. In study 1, mice were anesthetized with ketamine/xylazine, and a baseline blood sample was obtained via the retro-orbital plexus. Mice were then injected via the retro-orbital plexus with 0.2 or 1.0 mg of LDL protein. Blood samples were taken at 15 min and at 4, 8, and 24 h postinjection via the retro-orbital plexus. In study 2, mice were anesthetized and injected with 200 μ g of copper OxLDL protein, and blood samples were obtained at baseline and at 5 min and 0.5, 1, 2, 3, and 6 h postinjection. In both studies, the first blood sample was obtained from the retro-orbital plexus contralateral to that used for injection. In all experiments, EDTA was added to the blood immediately after collection and plasma was separated and frozen at -80°C. All procedures performed on the mice were in accordance with National Institutes of Health and institutional guidelines.

Immunoassays

Isolated plasma samples were analyzed for autoantibody titers, the relative content of hLDL, oxidation epitopes of LDL, and immune complexes to the injected LDL by chemiluminescent immunoassays using methods previously described (24). Autoantibodies were measured by plating MDA-LDL at 50 μ l/well and 5 μ g/ml PBS (containing 0.27 mM EDTA and 0.02% sodium azide) overnight at 4°C. After the addition of the preinjected mouse plasma at a 1:200 dilution in PBS containing 1% BSA (1% BSA/PBS), IgG and IgM autoantibodies were measured using alkaline phosphatase-labeled goat anti-mouse IgG and IgM (Sigma), respectively, diluted with 1% BSA in TBS containing 1 mM MgCl₂ and 1 mM ZnCl₂. After 1 h, LumiPhos 530 (Lumigen, Inc.) was diluted 1:1 with distilled water and 25 μ l was added to each well, followed by an incubation of 90 min at room temperature in the dark. Plates were analyzed for chemiluminescence in a Dynex Luminometer. Each plasma sample was assayed in triplicate, and results are expressed as relative light units (RLU) per 100 ms.

To determine the relative content of hLDL in the mouse plasma, MB47, a murine monoclonal antibody specific for human apoB-100 (25), was added to microtiter wells at a concentration of 5 μ g/ml in PBS overnight at 4°C. After washing the plate with an automated plate washer with PBS, all wells were blocked by the addition of 1% BSA/PBS for 45 min at room temperature. The plates were washed again, and 50 μ l of murine plasma, diluted 1:100 in 1% BSA/PBS, was added to wells for 1 h at room temperature. It was determined in preliminary experiments that the 1:100 dilution did not saturate the capacity of MB47 to bind apoB-100 under these conditions. To determine the relative amount of hLDL bound to each well, biotin-labeled goat anti-human apoB-100 (Bioscience International, Kennebunkport, ME) was added to each well for 1 h at room temperature, followed by

washing of the plates with TBS and the addition of an alkaline phosphatase-labeled NeutrAvidin (Pierce Biotechnologies) in TBS containing 1% BSA, 1 mM MgCl₂, and 1 mM ZnCl₂. Each plasma sample was assayed in triplicate, and results are expressed as RLU.

The relative amount of OxPL epitopes present on the captured LDL was determined using the IgM monoclonal antibody EO6 (20) that was biotin-labeled using EZ-Link Biotin-LC-Hydrazide (Pierce Biotechnologies). EO6 was added at 1.5 μg/ml and 50 μl/well in 1% BSA/TBS. The content of OxPL per apoB-100 particle (e.g., a measure of OxLDL) was then expressed as the ratio of EO6 binding (in RLU) divided by the content of anti-apoB-100 binding (in RLU). This value gives a relative content of OxPL per apoB-100 particle. To measure OxPL epitopes on the endogenous murine apoB-100-containing particles, the monoclonal antibody LF3 (26), specific for mouse apoB-100, was used to coat the microtiter well, whereas a biotin-labeled LF5 (26) monoclonal antibody to a noncompeting epitope of murine apoB-100 was used for detection as described above. LF3 and LF5 were kindly provided by Dr. Steve Young. The content of EO6/murine apoB-100 was calculated in a manner similar to that used for the EO6 content of human apoB-100.

To detect immune complexes associated with the circulating hLDL, human apoB-100 was captured using MB47 as described above, alkaline phosphatase-labeled goat anti-mouse IgM (Sigma) was used, and chemiluminescence was detected as described above. To measure the murine IgG present on the captured hLDL, F(ab')₂ fragments of the MB47 were produced (using the ImmunoPure kit from Pierce Biotechnologies) and used for plating to capture hLDL. For this assay, an alkaline phosphatase-labeled goat anti-mouse IgG-Fc antiserum was used for detection, which was shown in preliminary experiments not to bind to the plated MB47 F(ab')₂.

Statistical analyses

Statistical analyses were performed using StatView 5.0.1 software via ANOVA followed by the Bonferroni/Dunn post hoc test when more than two samples were analyzed at the same time point. The significance levels were set at $P < 0.05$.

RESULTS

Characterization of immune-competent and immune-incompetent mice

ApoE^{-/-} mice were crossed with RAG2^{-/-} mice to generate apoE^{-/-} mice that lacked mature T and B cells (18). Female apoE^{-/-} and apoE^{-/-}RAG2^{-/-} mice were maintained on chow diet or placed on a high-fat/cholesterol-enriched diet for a period of 2 months. Plasma cholesterol levels were determined 1 month after the initiation of the high-fat diet (Table 1), a time at which plasma cholesterol levels are already greatly increased. Note that both the apoE^{-/-} and the apoE^{-/-}RAG2^{-/-} mice on the high-fat/high-cholesterol diet had comparable and greatly increased plasma cholesterol levels, ~1,200–1,400 mg/dl. Although we did not check the cholesterol levels for the chow-fed apoE^{-/-} mice at this time point, data are presented for a comparable set of female animals (Table 1).

To validate that our apoE^{-/-}RAG2^{-/-} mice were deficient in plasma immunoglobulins, we examined the plasma of a subset of the mice used in this study for the presence of antibodies to MDA-LDL (Fig. 1). Immune-

TABLE 1. Plasma cholesterol levels of apoE^{-/-} and apoE^{-/-}RAG2^{-/-} mice

Mouse Strain	Diet	Plasma Cholesterol	n
		mg/dl	
apoE ^{-/-}	Chow	550 ± 26	12
apoE ^{-/-} RAG2 ^{-/-}	Chow	707 ± 48	11
apoE ^{-/-}	High fat	1,423 ± 87	15
apoE ^{-/-} RAG2 ^{-/-}	High fat	1,205 ± 120	18

apoE^{-/-}, apolipoprotein E-deficient; RAG2^{-/-}, recombination-activating gene 2-deficient. Fasting plasma cholesterol levels were determined in the mice after 1 month on the high-fat diet and in mice maintained on chow diet. Values shown are means ± SEM.

deficient mice did not exhibit any reactivity with MDA-LDL, whereas IgM (Fig. 1A) and IgG (Fig. 1B) reactivity against MDA-LDL was present in the plasma of all the immune-competent mice.

Clearance of native hLDL

To follow the oxidation of a discrete LDL particle and its clearance from the circulation, we injected native hLDL into immune-competent and immune-incompetent apoE^{-/-} mice fed chow or a high-fat/cholesterol diet. The availability of a monoclonal antibody that exclusively recognizes human apoB-100 (MB47) allowed us to use unlabeled hLDL as a tracer because it is possible to specifically immunocapture the hLDL from plasma obtained after injection into mice (27). Plasma was obtained serially after injection, and the relative concentration of human apoB-100 in plasma was determined immunochemically (Fig. 2A). The earliest time point, 15 min, shows essentially the same amount of human apoB-100 in all four groups of animals, verifying that nearly equal amounts of

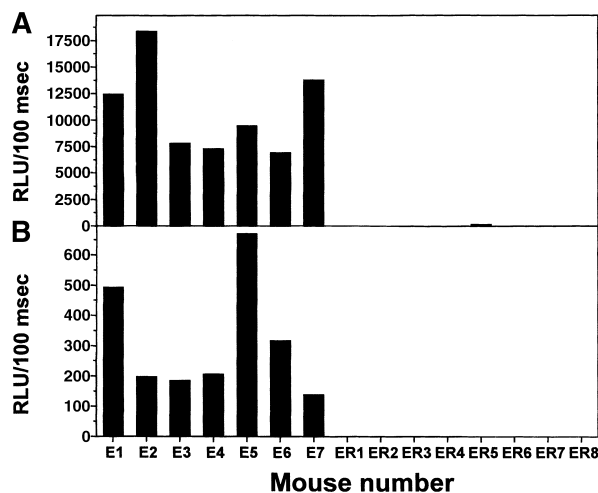


Fig. 1. Apolipoprotein E-deficient/recombination-activating gene 2-deficient (apoE^{-/-}RAG2^{-/-}) mice lack antibodies to malondialdehyde-modified LDL (MDA-LDL). The presence of IgM (A) and IgG (B) antibodies against MDA-LDL in the plasma of individual apoE^{-/-} (E1–E7) and apoE^{-/-}RAG2^{-/-} (ER1–ER8) mice fed the high-fat diet for 1 month is indicated. Antibody levels are expressed as relative light units (RLU)/100 ms.

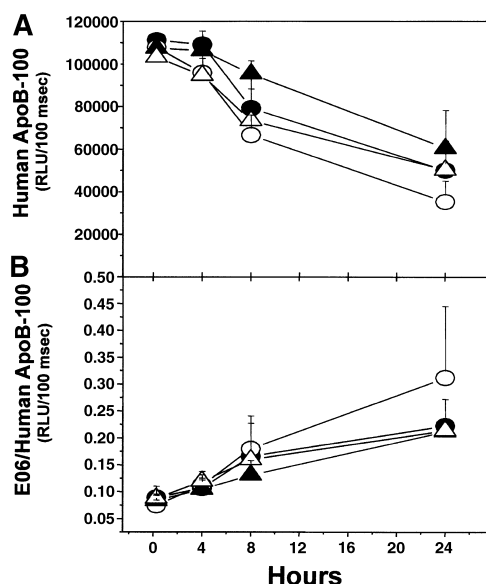


Fig. 2. Clearance and oxidation of native human LDL (hLDL) after injection into apoE^{-/-} mice. Four month old female apoE^{-/-} and apoE^{-/-}-RAG2^{-/-} mice maintained on chow diet or fed a high-fat/cholesterol diet for 8 weeks were injected with 1 mg of hLDL. The mice were then bled at 0.25, 4, 8, and 24 h postinjection. The relative levels of human apoB-100 in the plasma (A) and E06 epitopes per apoB-100 on the hLDL (B) at each time point were determined by immunoassay. The means \pm SD for 5 chow-fed apoE^{-/-} mice (closed circles), 10 chow-fed apoE^{-/-}-RAG2^{-/-} mice (open circles), 6 diet-fed apoE^{-/-} mice (closed triangles), and 10 diet-fed apoE^{-/-}-RAG2^{-/-} mice (open triangles) are shown. Whether fed chow or the high-fat diet, human apoB-100 levels were higher in the immune-competent mice at all time points. However, human apoB-100 levels were significantly different between immune-competent and immune-incompetent mice only at 4 h for both chow-fed and diet-fed mice and at 8 h for diet-fed mice ($P < 0.01$).

hLDL had been injected intravenously into each animal group.

In all of the apoE^{-/-} mice, there was a clear time-dependent removal of human apoB-100 from the circulation, so that by 24 h there was only 30–60% of the injected hLDL remaining in the plasma. Because these animals are wild type for the LDL receptor, this clearance is expected and is similar to the clearance reported by Shih et al (27). Even though hLDL is not as well recognized by the murine LDL receptor, the latter is nevertheless a major pathway for the clearance of hLDL (28). If immune-dependent mechanisms were to account for a significant fraction of the clearance, the apoE^{-/-}-RAG2^{-/-} mice should exhibit delayed clearance of the injected hLDL compared with the clearance in immune-competent mice. However, the reverse was observed. At both 4 and 8 h, the immune-deficient apoE^{-/-}-RAG2^{-/-} animals fed the high-fat/cholesterol diet for 2 months cleared the hLDL to a greater extent than did the immune-competent apoE^{-/-} animals ($P < 0.01$). This was also true for the animals maintained on chow diets at the 4 h time point. These data were obtained after injection of 1.0 mg of hLDL into female mice, but similar results were obtained after injection of only 0.2 mg of hLDL and when such ex-

periments were performed in male mice and in mice backcrossed to C57BL/6 mice for 10 generations (data not shown).

Extent of in vivo oxidation of the injected native LDL (and/or acquisition of oxidized epitopes)

When LDL undergoes oxidation, one of the products is OxPL, which is retained within the lipoprotein. PC-containing OxPLs are recognized by natural IgM autoantibodies, such as the prototypic antibody EO6, that were cloned from the spleens of apoE^{-/-} mice (20). To determine if the injected hLDL acquired such OxPLs while in the circulation, we captured the hLDL in the plasma obtained at various time points after injection and used biotinylated EO6 to detect the content of PC-containing OxPLs on the hLDL (Fig. 2B). There was a clear increase in EO6 reactivity per apoB-100 particle between 0 and 24 h, whether the apoE^{-/-} mice were maintained on chow or a high-fat diet or whether they were immune competent or immune incompetent. Indeed, there was little difference between the pairs of observations based upon immune status.

We next asked whether we could detect immune complexes containing the injected hLDL and endogenous murine immunoglobulins after injection into immune-competent mice. This was accomplished by analyzing the content of murine IgM and IgG bound to the captured hLDL, as described in Experimental Procedures. Immune complexes with the hLDL were observed in all immune-competent mice (Fig. 3), and the levels of these immune complexes increased with time after injection of LDL. This suggests that the bound immunoglobulins most likely recognized modifications of the injected LDL that were increasing with time of circulation. (Note that these data too are expressed as Ig bound per LDL particle and thus represent the cumulative content of Ig bound to the hLDL over time.) More immune complex formation with

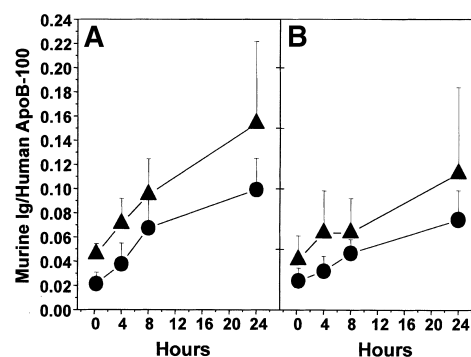


Fig. 3. Immune complexes are associated with the injected hLDL in immune-competent mice. The plasma obtained from the experiment described in Fig. 2 was assayed for the presence of murine IgM (A) and IgG (B) on the hLDL injected into chow-fed apoE^{-/-} mice (circles) and high-fat diet-fed apoE^{-/-} mice (triangles) by immunoassay after capture of the hLDL. Data shown are ratios of the binding of IgM or IgG divided by the amount of human apoB-100, as described in Experimental Procedures. IgM levels were significantly higher in high-fat diet-fed apoE^{-/-} mice except at the 24 h time point. Error bars represent SD of each of the ratios.

the hLDL was seen in the high-fat-fed apoE^{-/-} mice compared with the chow-fed mice.

Clearance of oxidized hLDL

The recognition of OxLDL by scavenger receptors requires a high degree of modification of the LDL. At the lower levels of lysine modification of apoB-100 that occur in so-called minimally modified LDL, the LDL is still LDL receptor competent and is fully capable of binding to and being endocytosed by the LDL receptor pathway (29). Undoubtedly, the degree of modification occurring on the injected native hLDL described in the experiments above is quite small, and such hLDL is truly "minimally modified." Whether this dependence on the extent of modulation of LDL might also apply to immune clearance is not known. It is well known that it is very difficult, if not impossible, to generate extensively modified LDL in the presence of plasma; therefore, we reasoned that the studies described above might not have revealed a significant impact of immune deficiency on LDL metabolism because of the insufficient extent of LDL modification that occurred to the injected hLDL. Accordingly, we undertook analogous studies, but this time instead of injecting native hLDL we injected hLDL that had been extensively oxidized *ex vivo* before the time of injection (Fig. 4). Copper OxLDL was prepared and shown to be extensively modified, as demonstrated by altered electrophoretic mo-

bility on agarose gels and by a greatly increased content of EO6 reactivity (data not shown). These human OxLDL samples were then injected into mice maintained on the high-fat diet. The clearance of the injected human OxLDL was monitored by capture with MB47. MB47 is capable of binding the apoB of OxLDL unless the apoB is extensively degraded, and preliminary studies demonstrated the capacity of MB47 to bind the OxLDL *in vitro* as well as in the murine plasma after injection. We also measured the EO6 immunoreactivity on the captured hLDL. Because of the known rapid clearance of OxLDL, we performed preliminary experiments in wild-type mice with injected human OxLDL and determined that the time frame of these experiments should be reduced from 24 to 6 h, by which time ~60–90% of the injected dose had been cleared from the circulation, whether determined by the disappearance of human apoB-100 or EO6 epitopes. When the human OxLDL was injected into the murine models, we noted that, compared with the immune-competent mice, the clearance of the human apoB-100 was actually somewhat delayed in the first hour after injection in the immune-deficient mice (Fig. 4A). However, after the first hour of clearance, the remaining clearance trajectory was similar in immune-deficient and immune-competent mice. Because the absolute EO6 immunoreactivity declined faster than that of the human apoB-100, the EO6/apoB-100 ratio showed a steady decline from 1.0 to 0.2 in plasma aliquots drawn over time in both immune-competent and immune-incompetent mice (Fig. 4B). One interpretation of these data is that among the human OxLDL particles injected there is considerable heterogeneity in the degree of oxidation and that the most heavily oxidized particles are cleared more rapidly in the immune-competent apoE^{-/-} mice. Overall, these data suggest that even in this parameter there was little difference by immune status.

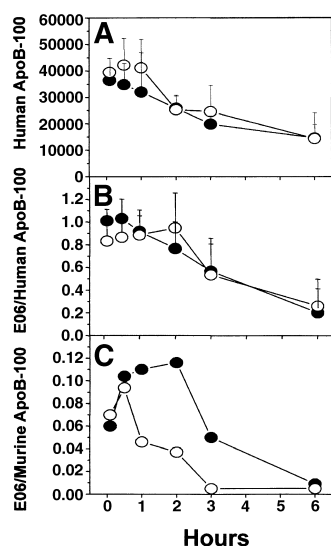


Fig. 4. Clearance of oxidized LDL. Four month old female apoE^{-/-} and apoE^{-/-}RAG2^{-/-} mice fed a high-fat diet for 8 weeks were bled at time 0 and injected with 0.2 mg of oxidized hLDL. The mice were then bled at 5 min, 30 min, and 1, 2, 3, and 6 h postinjection. A: Relative levels of human apoB-100 in plasma over time. B: Ratio of EO6 epitopes per human apoB-100 over time in the same samples. Note that the ratio is decreasing, indicating a more rapid clearance of the oxidized phospholipid detected by EO6 compared with apoB-100. C: Appearance of EO6 epitopes on the murine apoB-100 in the same samples. EO6 epitopes on human and murine apoB-100 were determined by immunoassay as described in Experimental Procedures. The values shown are means \pm SD for apoE^{-/-} mice (closed circles) and apoE^{-/-}RAG2^{-/-} mice (open circles) ($n = 8$ for both strains).

Transfer of oxidized lipid from hLDL to mouse LDL

One factor that could confound the results of these experiments is the transfer of OxPL from the injected hLDL to the endogenous mouse LDL. If this occurred to a significant extent, it would underestimate the ratio of EO6/apoB-100 because the assay depends upon the initial capture of only human apoB-100. To assess the extent of transfer, mouse apoB-100-containing lipoproteins were captured using monoclonal antibody LF3, which exclusively binds to murine apoB-100 and not to human apoB-100 (26). EO6 reactivity associated with the mouse apoB-100 particles was assessed (Fig. 4C). Although there was clearly a small amount of transfer of oxidized lipid to murine LDL (note the different scales in Fig. 4B, C), there was no difference in the extent of transfer of oxidized lipid to the endogenous LDL as a function of immune status.

DISCUSSION

Atherosclerosis is accompanied by proinflammatory mechanisms and by the activation of innate and acquired immune responses. In hypercholesterolemic animal mod-

els, there is a marked humoral response to oxidation-specific epitopes of OxLDL that parallels the development of atherosclerosis (9, 10). The suggestion that this immune response can modulate atherogenesis is inferred from the numerous studies now demonstrating that immunization of hypercholesterolemic animals with OxLDL (or with models of oxidation-specific epitopes found on OxLDL, such as MDA-LDL) leads to an amelioration of the progression of disease (11–15). However, the mechanism(s) by which this occurs remains undefined.

One mechanism by which high autoantibody titers to OxLDL epitopes could be beneficial is by binding to circulating forms of OxLDL, effecting enhanced removal to organs rich in macrophages, such as liver, spleen, bone marrow, and lungs, thus diverting minimally oxidized forms away from the artery wall. Experimental evidence of such antibody-mediated enhanced plasma clearance was previously provided by the demonstration that there was enhanced plasma clearance of glycated LDL in rabbits immunized with glycated LDL and that the glycated LDL was diverted to the reticuloendothelial system and away from the artery (16).

In the current studies, we used a novel immunological strategy to trace the clearance of LDL in mice. In confirmation of previous reports (27, 28), we demonstrate the rapid plasma clearance of hLDL in apoE^{-/-} mice. Despite this rapid clearance, there was a progressive accumulation of OxPL epitopes on the LDL during its residence in the circulation. It is unclear from these studies whether the progressive accumulation of OxPL on LDL represents de novo oxidation that occurred as the LDL passed through tissues or whether it represents the transfer onto LDL of oxidized lipids from other sources, such as other peroxidized lipoproteins or even cells undergoing apoptosis or necrosis, which we have shown to contain such epitopes (30). In this regard, it is of considerable interest that whereas we reproducibly observed a progressive increase in EO6 epitopes over time on the hLDL injected into hypercholesterolemic apoE^{-/-} mice (Fig. 2), in preliminary studies we observed little if any such increase in LDL injected into equally hypercholesterolemic LDL receptor-deficient mice, even though the residence time of the hLDL in these mice was greatly prolonged (data not shown). Thus, 24 h after injection of hLDL into LDL receptor-deficient mice, less than 10% of the injected lipoprotein had disappeared from the plasma and the retained lipoprotein was much more modestly oxidized (the EO6/apoB-100 ratio was 0.07–0.10 compared with 0.15–0.25 for apoE^{-/-} mice; Fig. 2). These observations would support previous suggestions that there is a proportionately increased degree of oxidation in the apoE^{-/-} mice (31, 32). The “antioxidant” properties of apoE may account for the apparently higher rate of accumulation of EO6 reactivity on hLDL in apoE^{-/-} mice compared with the absence of this accumulation in LDL receptor-deficient mice.

In this study, we injected hLDL into apoE^{-/-} mice that had high titers of autoantibodies to OxLDL, as assayed by anti-MDA-LDL antibodies. Our working hypothesis was

that as the hLDL became progressively modified, it would bind the circulating antioxidation-specific antibodies, and in turn this would accelerate the clearance of the minimally modified LDL from plasma. Consequently, we predicted that the hLDL would have an enhanced rate of clearance in the immune-competent mice compared with the apoE^{-/-}RAG2^{-/-} mice, which lacked such antibodies. In contrast to our original hypothesis, however, our data show that the clearance of the modified LDL by immune-mediated mechanisms appears to play little if any role in the removal of bulk LDL from plasma. The clearance of the injected LDL probably occurs via a variety of mechanisms. First, the classic LDL receptor pathway is presumably responsible for the rapid clearance of the bulk of the native LDL, possibly because of decreased competition attributable to the absence of apoE (28). The extent of modification of the bulk of injected hLDL is not likely to be sufficient in and of itself to mediate scavenger receptor-mediated clearance, although we cannot rule out the possibility that a small fraction of particles were sufficiently modified to have such enhanced clearance. Another possibility that we had anticipated was that of immune-mediated removal of such heavily modified particles as a consequence of immunoglobulin binding and consequent Fc-mediated clearance. Indeed, as the hLDL circulated it acquired an increasing burden of immunoglobulin in the immune-competent apoE^{-/-} mice (Fig. 3), and the presence or absence of these immune complexes had little impact on the overall rate of LDL clearance, suggesting that these immune complexes did not play a major role in the removal of the minimally modified hLDL via Fc-mediated clearance. To the contrary, if anything, we noted that in the immune-deficient mice there was possibly an accelerated removal of human apoB-100 from the plasma. The mechanism that accounts for this observation is not at all clear. One interpretation is that the formation of immune complexes with LDL in the plasma of immune-competent mice actually “traps” this modified hLDL in the plasma compartment, delaying its normal clearance.

In the studies described above, we traced the metabolic behavior of hLDL in which a small fraction of the particles became minimally modified. It might be argued that the degree of modification was insufficient to attract sufficient immunoglobulin binding to effect enhanced plasma clearance. Therefore, we generated an extensively OxLDL by incubation with copper. These extensively modified particles contain OxPL epitopes recognized by EO6 in both the lipid phase and covalently bound to the apoB-100 and up to 80 mol of oxidized PC-containing phospholipids covalently bound to the apoB-100 (33). Thus, one would expect such a modified LDL to show enhanced clearance when injected into the immune-competent apoE^{-/-} mice if immunoglobulin-mediated clearance was relevant. As expected, the absolute rate of clearance of the OxLDL, as determined by human apoB-100 levels, was substantially accelerated compared with the clearance of native hLDL, presumably because of scavenger receptor-mediated clearance of this extensively modified LDL (Fig.

4A). However, again we did not observe any substantial difference in the rate of clearance of the human apoB-100 of OxLDL between the immune-competent and immune-incompetent apoE^{-/-} mice. We also studied the rate of clearance of the OxPL detected by EO6 expressed per LDL particle (Fig. 4B). Note that over time there is a steady decrease in the EO6/apoB-100 ratio. This was unexpected, for there is up to 80 mol of oxidized PC-containing phospholipids covalently bound to apoB-100 in such OxLDL, and one might have expected the EO6/apoB-100 ratio to remain near unity as the apoB-100 was cleared from plasma. The decline in this ratio over time is compatible with several possibilities. First, the temporal change in the ratio of EO6 to apoB-100 after injection could be compatible with the heterogeneity of clearance of the particles (e.g., some particles were more heavily oxidized than others and these were most rapidly removed, leading to the declining EO6/apoB-100 ratio of those particles remaining in plasma). Scavenger receptor-mediated clearance mechanisms would clearly favor the most extensively modified lipoprotein particles. This is consistent with the known interaction of scavenger receptors with OxLDL (34). Another possibility contributing to the decline in the OxPL/apoB-100 ratio could be that a portion of the EO6 reactivity in the OxLDL that is in the lipid phase (33) is transferred off the OxLDL to other tissues and other lipoproteins. To examine this possibility, we used the same immunological capture technique to capture the endogenous murine apoB-100 particles in the plasma samples. As shown in Fig. 4C, there was a steady increase in the content of OxPL per murine apoB-100 during the first few hours after injection of the human OxLDL, followed by a decay as presumably these murine lipoproteins were cleared. The time course of the appearance and then decay of the OxPL on murine apoB-100 particles, in relationship to the disappearance of OxPL from human apoB-100 particles, suggests a typical precursor product relationship between the disappearance of the EO6 reactivity from the injected human OxLDL and its appearance in the murine apoB-100 particles. However, the amount of OxPL detected on the murine apoB-100 particles was very small (e.g., note that the EO6/apoB-100 ratio in the injected human OxLDL was 1.0 and the maximal accumulation in the murine apoB-100 particles was only 0.1). Making a number of assumptions about the pool sizes of the injected human OxLDL and of the murine apoB-100 particles, we calculated that such transfer could account for no more than a few percent of the excess OxPL that initially entered plasma associated with the human OxLDL. Nevertheless, these data demonstrate the potential for transfer of at least some of the OxPL on OxLDL to other lipoprotein acceptors, and we speculate that a much greater fraction was transferred to other tissue acceptors.

Although the overall rate of clearance of the OxLDL in the immune-competent and immune-incompetent mice was not different, a careful inspection of the decay curves reveals a small difference in the behavior of OxLDL between the two mice. Clearance was rapid in the first hour,

particularly at the 60 min time point, but was much slower in the immune-deficient recipient. Two possible explanations are that immune clearance may play a role in the removal of the most extensively OxLDL or that the absolute expression of scavenger receptors may be lower in immune-deficient mice because of the absence of immune cells expressing such receptors. These data emphasize the complexity of interpreting metabolic clearance curves.

There are several possible explanations for the observation of a continuous increase in the EO6/human apoB-100 ratio in the plasma of animals injected with native LDL (Fig. 2). The injected LDL may be "oxidized" in the recipient *in vivo*. Alternatively, OxPL may be transferred to the injected LDL from endogenous lipoproteins or cells of the recipient mouse. Because hLDL is probably less well recognized by the murine LDL receptor, perhaps modified LDL is less well cleared than native LDL, whether by the LDL receptor or by other clearance pathways. We cannot determine which of these possibilities plays the major role in accounting for our results. Presumably, at this minimum level of acquisition of oxidation epitopes, the modified LDL is not recognized by scavenger receptors. On the other hand, when we studied heavily oxidized hLDL (oxidized *ex vivo*), we observed the opposite phenomenon. As expected, the most heavily OxLDL appeared to be cleared more rapidly than the less oxidized particles. It is likely that scavenger receptors are involved prominently in this clearance. There may be a retardation of the clearance of the most heavily OxLDL in the immune-deficient animals at the earliest time points, but the basis for this is unknown, as discussed above.

In conclusion, comparison of the metabolism of native hLDL and substantially oxidized hLDL in apoE^{-/-} mice, either immune competent or immune incompetent, provides limited, if any, evidence of immune-mediated clearance of either minimally or even heavily OxLDL from the plasma. The data also indicate that the clearance mechanisms are sensitive to the extent of oxidative modification. Finally, this study describes a new model for the *in vivo* study of the metabolism of oxidatively modified hLDL, using the mouse as a reporter animal.

Although our data provide little support for the importance of antibodies to oxidation epitopes as clearance mechanisms for the removal of plasma LDL, they say little about the role of oxidation of lipoproteins in the process of atherogenesis or the possible implication of immune complexes being taken up via the Fc receptor on macrophages in the formation of lesion foam cells. In addition, some autoantibodies to certain oxidation epitopes may attenuate the process of atherogenesis by blocking the uptake by macrophages of OxLDL (35). Thus, the interactions of OxLDL with its cognate antibodies within the vessel wall could nevertheless be of great significance. ■

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