Turnover and tissue sites of degradation of glucosylated low density lipoprotein in normal and immunized rabbits

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Abstract Immunological mechanisms have been implicated in the atherogenic process since immunoglobulins are frequently found in the atherosclerotic aorta. We have previously shown that modifications of homologous low density lipoproteins (LDL) make it immunogenic. In particular we have demonstrated that immunization with homologous nonenzymatically glucosylated LDL (glcLDL) results in the generation of antibodies specific to the derivatized lysine residue, and that such antibodies do not react with native LDL epitopes. In the present study we immunized rabbits with reductively glucosylated rabbit LDL and then determined the effects of the circulating antibodies on the rates of plasma clearance and on the sites of degradation of LDL in which varying degrees of glucosylation had been achieved. In normal chow-fed animals, the plasma clearance of glcLDL was retarded in proportion to the extent of lysine derivatization. In contrast, in immunized animals the clearance of glcLDL was greatly accelerated. When 10% or more of lysine residues were derivatized, clearance of glcLDL was accelerated 50- to 100-fold. Even when only 5% of lysines were derivatized, plasma clearance was accelerated 2- to 3-fold. Cholesterol feeding inhibited LDL clearance from plasma and decreased LDL uptake of LDL receptor-rich tissues. In a similar manner, glucosylation of LDL inhibited its ability to bind to the LDL receptor and redirected sites of LDL degradation away from LDL receptor-rich tissues. Thus degradation of glcLDL by liver and adrenal was markedly diminished. The presence of antibodies to glcLDL also redirected sites of degradation of the modified LDL, primarily to the reticuloendothelial cells of the liver. There was no evidence for specific targeting of glcLDL–immunoglobulin complexes to the aorta; instead they were targeted to the liver. These data suggest that the presence of humoral antibodies to modified LDL acts to rapidly remove such LDL from plasma and specifically targets such complexes to reticuloendothelial cells, primarily in the liver. In this manner such antibodies may serve a useful purpose.— **Wiklund, O., J. L. Witztum, T. E. Carew, R. C. Pittman, R. L. Elam, and D. Steinberg.** Turnover and tissue sites of degradation of glucosylated low density lipoprotein in normal and immunized rabbits. *J. Lipid Res.* 1987. **28:** 1098–1109.

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Plasma low density lipoproteins (LDL) are a major source for the cholesterol deposited in the arterial wall during the development of atherosclerosis. Abnormalities in the metabolism of LDL and shifts in its sites of degradation therefore may be of great importance for the development of atherosclerotic lesions. Immunological mechanisms that modify LDL catabolism have been discussed by several investigators. In so-called "autoimmune hyperlipidemia" or in association with paraproteinemia, autoantibodies against LDL have been demonstrated (1–6). Such antibodies have also been found frequently in other states, such as diabetes and uremia (7, 8). An influence of antiLDL antibodies on lipoprotein metabolism has also been suggested (3, 5).

It has been suggested that immunological mechanisms may be of importance for the development of atherosclerosis. Depositions of immunoglobulins have been shown in atherosclerotic plaques (9, 10), possibly as a consequence of immune-complex deposition. This hypothesis is further supported by an increased occurrence of circulating immune-complexes in patients with various atherosclerotic disorders (11–14). We have previously shown that subtle modifications of homologous LDL render it immunogenic in guinea pigs (15, 16) and mice (17). One such modification is nonenzymatic glucosylation of the lysine residues of apoB. Recently we also showed the presence of antibodies against reductively glucosylated LDL (glcLDL) in human diabetic subjects (18).

In the present study we sought to investigate how the presence of circulating antibodies changes the metabolism

Abbreviations: LDL, low density lipoproteins; glcLDL, glucosylated LDL; TC, tyramine-cellobiose; PBS, phosphate-buffered saline; FCR, fractional catabolic rate.

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and the sites of degradation of glcLDL in an animal. This was done with special attention to degradation in the arterial wall. To accomplish this we studied metabolism of glcLDL in rabbits using methods previously developed in this laboratory for determining in detail the organs responsible for removal of LDL from plasma in rabbits (19). In this study we show that the presence of humoral antibodies to modified LDL profoundly alters its rate of clearance from plasma as well as its sites of degradation.

MATERIALS AND METHODS

Lipoprotein preparation and labeling

LDL (d 1.020–1.063 g/ml) was prepared from fresh rabbit plasma containing disodium EDTA (1 mg/ml) by standard ultracentrifugal techniques as previously described (20). Most preparations were divided into two aliquots and labeled with different radioiodine isotopes.

For turnover studies, LDL protein was iodinated with carrier-free ^{125}I or ^{131}I (Amersham) using 1,3,4,6,-tetrachloro-3,6-diphenyl-glycouril (Iodogen, Pierce Chemical Co.) (20). Unbound iodine was removed by extensive dialysis against phosphate-buffered saline (PBS). Specific activity ranged from 100 to 300 cpm/ng protein. Trichloroacetic acid (TCA) precipitability was greater than 98%, and less than 3–5% of the radioactivity was extracted into organic solvents.

For studies of the tissue sites of degradation, LDL was labeled with radioiodinated tyramine-cellobiose (TC) as previously described (21). Briefly, the TC adduct (0.1 μmol of TC for each 10 mg of LDL protein to be labeled) was iodinated with carrier-free ^{125}I or ^{131}I. The labeled adduct was then activated by addition of 1 mole-equiv. (0.1 μmol for each mg of LDL protein) of the cross-linking agent, cyanuric chloride. The activated ligand was used immediately to bind LDL in a solution, adjusted to pH 9.5 by addition of 0.3 M borate buffer. Preparations were then exhaustively dialyzed against PBS. Specific activity ranged from 100 to 760 cpm/ng protein.

Glucosylation of LDL

Reductive, nonenzymatic glucosylation of the iodinated LDL preparations was performed as described earlier (22). Briefly, sterile LDL was incubated at 37°C in PBS in the presence of 80 mM glucose and $NaCNBH_3$ (12.5 mg/ml). By varying the incubation times, the degree of derivatization of the lysine residues of LDL could be varied from 2% to 50% (23). Incubation of LDL for 6 hr yielded a preparation of LDL in which 3–5% of the lysine residues were derivatized, while incubation for 20, 36, and 72 hr caused 10, 15, and 25%, respectively, of lysine residues to be glucosylated (23). Incubation for 168 hr derivatizes more than 40–50% of the lysine residues.

Animal studies

New Zealand White (NZW) rabbits weighing 2.1–3.1 kg were maintained on normal rabbit chow (Purina), except in one experiment in which rabbits were fed regular chow supplemented with 2% cholesterol for 4 months prior to the experiments. At the time of the study, plasma cholesterol levels for these cholesterol-fed rabbits averaged 2,055 mg/dl (range 1,828–2,354 mg/dl).

Indwelling Silastic catheters were placed in the external jugular vein of the subject rabbits under general anesthesia (xylazine, 5 mg/kg, and ketamine, 35 mg/kg). Catheters were used for injection of labeled LDL and blood sampling. In studies using conventionally iodinated LDL, $2–3 \times 10^6$ counts (~15 μg of LDL protein) of each label were injected; for studies with TC-labeled LDL, $100–500 \times 10^6$ counts (~300 μg of LDL protein) were injected. Thyroidal sequestration of radioiodide was prevented by injection of 3 mg of NaI just before injection of the labeled LDL. Plasma decay kinetics were determined in terms of TCA-precipitable radioactivity in plasma, measured in a double-channel gamma spectrometer (LKB 1282, Compugamma). At the termination of the experiments in which TC-labeled LDL had been injected, after 24 hr unless otherwise stated, the rabbits were anesthetized and the systemic circulation was perfused at 100 mm Hg pressure using phosphate-buffered saline (PBS) (20). After perfusion the aorta was immediately removed and loose adventitial tissue was dissected away. Other organs were then removed in toto. Large or diffuse organs, such as skin, skeletal muscle, or bone marrow, were sampled in multiple sites. The estimated total weights of the latter organs were derived from organ to body weight ratios reported in the literature, as previously described (19). Thus, skeletal muscle, skin, bone marrow, and adipose tissue were assumed to represent 43.9, 13.4, 1.05, and 7.3% of total body weight, respectively. For smaller organs the whole organ was counted in a double-channel gamma counter, while larger organs were homogenized in water with a Brinkman Polytron (Brinkman Instruments Westbury, NJ) and an aliquot was counted. In addition to the tissues reported in Tables 2 through 4, we also sampled urinary bladder, heart, lymph nodes, ovaries, pancreas, and thymus. In aggregate these organs accounted for less than 1% of degradation and their data are not reported further, except for the ovarian data which are presented in Fig. 6. Data for intestine represent the sum of the values measured in stomach, small intestine, cecum, and large intestine. In every case more than 81% of the injected dose could be accounted for (in tissues, urine, feces, plasma samples, and residual radioactivity in plasma at time the animals were killed) and for all animals recovery averaged $93.4 \pm 6.0\%$ for TC-labeled glcLDL tracers and $92.8 \pm 8.6\%$ for the TC-labeled *native* LDL tracers.

Immunization and determination of antibody titers

For immunization, rabbit LDL was prepared and reductively glucosylated for 7 days as described above. For the primary immunization, about 100 μg of glcLDL protein with an equal volume of Freund's complete adjuvant was injected subcutaneously into both inguinal regions. The rabbits were then boosted two or three times with the same dose of glcLDL in Freund's incomplete adjuvant. The immunized, cholesterol-fed rabbits were given the primary injection after approximately 2 months on the cholesterol diet, and two booster doses were given about 8 and 5 weeks before the experiments. Plasma antibody titers were determined by solid phase radioimmunoassay as earlier described (15). Fifty ng of rabbit-glcLDL was immobilized in each well of 96-well polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, VA) by incubating 500 ng/well of glcLDL dissolved in 50 μl of PBS–0.01% EDTA for 2 hr at 37°C. Each well was then aspirated and washed four times with buffer (PBS–0.01% EDTA–0.02% NaN_3–0.05% Tween 20–0.1% bovine serum albumin–0.0015% Trasylol). To block remaining adsorptive sites on the plastic wells, each plate was then incubated with a post-coat buffer (PBS–0.01% EDTA–3% normal goat serum–3% bovine serum albumin) at room temperature for 30 min and aspirated to dryness. To measure the antibody titer, 50 μl of various dilutions of rabbit plasma were added to antigen-coated wells and plates were incubated for 18 hr at 4°C. Plasma was then aspirated, each well was washed four times, and the amount of immunoglobulin bound to the plates was quantitated by incubation with iodinated goat anti-rabbit immunoglobulin (Calbiochem, La Jolla, CA). The goat anti-rabbit immunoglobulin was iodinated using immobilized lactoperoxidase (Enzymobeads, Bio-Rad, Richmond, CA). Fifty μl of secondary antibody (approximately 0.5 μg/ml and $2.0–3.0 \times 10^5$ cpm) was added to each well and plates were incubated for an additional 4 hr at 4°C. Each well was then aspirated, washed four times, and bound radioactivity was quantified. A titer is defined as the reciprocal of that dilution of plasma giving binding of secondary antibody three times that of nonimmune plasma.

Data analysis

Plasma decay curves of radioiodinated LDL tracers were analyzed using an interactive curve peeling program, as previously described (20, 24). Computer analysis was used to fit a least-square multiexponential curve to each set of turnover data and to calculate fractional catabolic rates.

The number of counts recovered from each organ was related to the total recovered counts from all sources except plasma. Results could thus be expressed as % of total degraded tracer accounted for by each tissue, or per gram of each tissue (19). When different animals and degradation sites of different tracers were compared, we also performed calculations that took into account differences in FCR of the various tracers. The results from these calculations were expressed as the fraction of the plasma pool degraded per gram of tissue, i.e., the FCR/g (19).

RESULTS

Immunization of rabbits with homologous glcLDL

In previous studies immunization of guinea pigs or mice with glcLDL made using homologous LDL resulted in antibodies that were directed against glcLDL but *not* against *native* LDL (15, 16). In a similar manner, in the present study, each rabbit immunized with rabbit glcLDL showed a positive antibody titer specific to glcLDL. However, the immune response was variable so that at the time the rabbits were used in experiments, some had a very high titer (over 20,000) while others showed a much lower titer (e.g., between 800 and 2,000). However, no effect of titer was evidenced in the studies presented in this report. Cholesterol feeding did not interfere with antibody response.

Turnover of glucosylated-LDL in control, immunized and cholesterol-fed rabbits

We have previously shown that extensive glucosylation of LDL inhibits its binding to the LDL receptor and thus delays its clearance from plasma (22). In guinea pigs we also showed that this delay was directly related to the degree of glucosylation (23). In the present study these observations could be confirmed in rabbits. In **Table 1** the FCR is given in relation to that of *native* LDL. In the control, nonimmunized rabbits, the FCR was inversely related to the degree of glucosylation. When approximately 50% of LDL lysine residues were derivatized, glcLDL(50%), the FCR was only 17% that of *native* LDL **(Fig.1)**; derivatization of 5% of lysine residues, glcLDL(5%), resulted in a consistent reduction of FCR by 15–20% **(Fig. 2)**. In contrast, in the *immunized* rabbits the clearance of all glcLDL preparations was accelerated. Thus, when only 5% of lysine residues was derivatized, the glcLDL(5%) tracer was accelerated 2- to 3-fold. When 10% or more of lysine residues was glucosylated, the clearance of the tracer was greatly accelerated, achieving rates 50- to 100-fold or more greater than the native LDL tracer. It should also be noted that whereas the rapid clearance of the glcLDL(50%) tracer in the immunized rabbit would be recognized as distinctly abnormal by simple inspection of the plasma decay curves (Fig. 1), the plasma decay curve of glcLDL(5%) in the immunized rabbit (Fig. 2) does not appear abnormal and the true etiology of its enhanced clearance would not likely be appreciated from the shape of the curve.

TABLE 1. Relative rates of clearance of LDL preparations with varying degrees of glucosylation in nonimmunized control rabbits and in immunized rabbits

Tracer	Control	Immunized
Chow-fed		
Native LDL	1.00	1.00
glcLDL (5%)	0.82 ± 0.10 (n = 3)	2.91 ± 0.35 (n = 5)
glcLDL (10%)	0.56 (n = 1)	113.0 ± 5.6 (n = 2)
glcLDL (15%)		104.0 (n = 1)
glcLDL (25%)		55.0 (n = 1)
glcLDL (50%)	0.17 ± 0.01 (n = 2)	89.0 ± 58 (n = 2)
Cholesterol-fed		
Native LDL	0.42 ± 0.03 (n = 2)	0.41 ± 0.01 (n = 2)
glcLDL (5%)	0.38 ± 0.06 (n = 2)	4.06 ± 2.7 (n = 2)

Shown are relative rates of clearance of various LDL preparations injected into chow-fed and cholesterol-fed rabbits that were non-immunized controls or immunized. Fractional catabolic rates (FCR) were calculated as explained in Methods and expressed as a fraction of the FCR found for native LDL under similar conditions. The absolute FCR for native LDL in control animals was 0.070 ± 0.007 pools/hr (n = 2) and the FCR for native LDL in immunized animals was 0.076 ± 0.003 pools/hr (n = 3).

In the cholesterol-fed rabbits the FCR for *native* LDL was reduced to 42% of that observed for native LDL in chow-fed animals (Table 1). The clearance of glcLDL (5%) in the cholesterol fed rabbits was also reduced to 38% of that seen for native LDL. These data are consistent with the known ability of cholesterol feeding to inhibit LDL receptor activity (25). In the cholesterol fed *and* immunized rabbits, the plasma disappearance of *native* LDL was similar to that observed with cholesterol feeding alone (Table 1 and **Fig. 3**). However, in marked contrast, the rate of clearance of glcLDL(5%) was enhanced 5- to 10-fold over that of *native* LDL in these rabbits, obviously due to the *immune*-mediated clearance (Fig. 3), i.e., the marked hypercholesterolemia in these animals did not prevent the immune recognition of glcLDL.

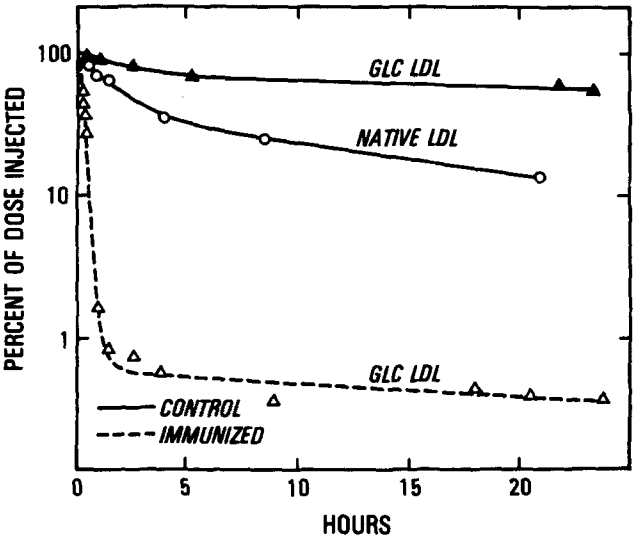


Fig. 1. Plasma radioactivity decay curves for 50% glucosylated LDL (glcLDL) (△, ▲) and native LDL (○) injected into normal rabbits and immunized rabbits. ^{125}I-Labeled TC-LDL or ^{125}I-labeled TC-glcLDL(50%) was injected and serial blood samples were drawn from an ear vein. The radioactivity at each time point is expressed as the percent remaining in plasma of the initial dose.

Sites of degradation of native LDL in control, immunized, and cholesterol-fed rabbits

Table 2 shows the sites of degradation of *native* LDL in terms of the counts recovered in each organ as a percentage of total recovered counts. When expressed this way the data show the relative importance of tissue sites of degradation irrespective of differences in absolute catabolic rate (19). As shown in column A, the liver was the major site of LDL degradation in control rabbits, accounting for 58% of overall LDL degradation. Bone marrow, skin, and skeletal muscle were the next most active; their total contribution only accounted for an additional 17%. The tissues responsible for degradation of native LDL in immunized rabbits (Table 2, column B) were virtually identical, as expected, since the anti-glcLDL antibodies do not react with native LDL.

Cholesterol feeding significantly changed the degradation sites of native LDL (Table 2, columns C and D). The hepatic contribution was reduced to only 40–45% of total LDL degradation, while the degradation in skeletal muscle and skin was increased. The contribution of the bone marrow was also reduced. Though the contribution of the adrenal gland to overall LDL catabolism was small even in normal chow-fed rabbits, with cholesterol feeding its contribution was further reduced.

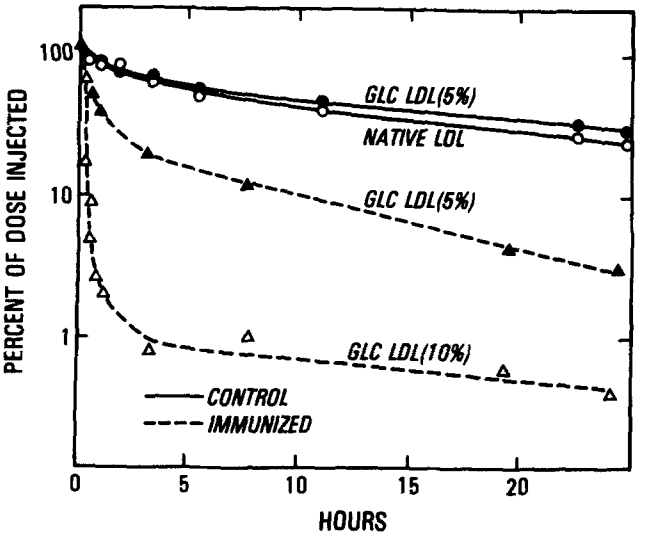


Fig. 2. Plasma radioactivity decay curves of native LDL and glcLDL(5% and 10%) in control and immunized rabbits. The radioactivity at each time point is expressed as the percent remaining in plasma of the initial dose.

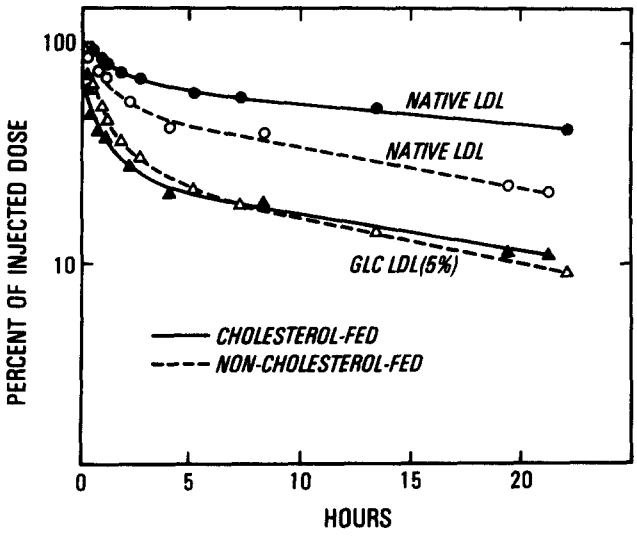


Fig. 3. Plasma radioactivity decay curves for native LDL and glcLDL(5%) simultaneously injected into two immunized rabbits. In one experiment native LDL (○) and glcLDL(5%) (△) were injected into a chow-fed rabbit and in the other experiment native LDL (●) and glcLDL(5%) (▲) were injected into a cholesterol-fed rabbit. Note that whereas cholesterol feeding slows the catabolism of native LDL, the presence of antibodies accelerates the clearance of glcLDL(5%) in both chow-fed and cholesterol-fed rabbits. The radioactivity at each time point is expressed as the percent remaining in plasma of initial dose.

Sites of degradation of glcLDL in nonimmune, chow-fed and cholesterol-fed rabbits

Table 3 presents sites of degradation of glcLDL in nonimmune chow-fed (columns B, and C) and cholesterol-fed rabbits (column D). Data for native LDL are again presented to facilitate comparisons (column A). Although the FCR for glcLDL (5%) tended to be less than that of native LDL, no differences could be detected in the relative uptake into various sites of degradation compared to native LDL (i.e., column A vs. column B). When a heavily glucosylated preparation, glcLDL (50%), was injected however, the liver was responsible for degradation of only 38% of total recovered. The amount degraded by the adrenals was reduced by 84%. Even though the contribution of aorta to overall glcLDL degradation was low, i.e., 0.03%, this value was still three-fold greater than that observed with native LDL in the limited number of animals in this study. The sites of degradation of glcLDL (5%) in cholesterol-fed rabbits were very similar to those observed for native LDL in cholesterol-fed rabbits (compare column D, Table 3 to columns C and D, Table 2).

Sites of degradation of glcLDL in immunized rabbits

As shown in Fig. 1, when heavily glucosylated LDL (glcLDL(50%)) was injected into an immunized rabbit, its plasma clearance was greatly accelerated and virtually all the tracer had been cleared from plasma within 30 min. In an initial study we killed an animal injected with a glcLDL(50%) tracer after 3 hr and examined tissues responsible for the clearance of the presumed glcLDL (50%)-immunoglobulin complexes (**Table 4**, column B). The liver was clearly the predominant site, accounting for 66% of recovered counts. The lung was the second most active organ while bone marrow and spleen accounted to a lesser extent. The accumulation in lung most likely reflected binding/entrapment of large immune complexes within the vasculature of the pulmonary bed. This interpretation is supported by the observation that in a second immunized rabbit, injected with the same glcLDL(50%) preparation but killed after 24 hr, the amount of label

TABLE 2. Sites of degradation of native LDL in rabbits. Percent contribution of individual organs to overall degradation

	A Controls (n = 2)	B Immunized (n = 3)	C Cholesterol-fed (n = 2)	C Cholesterol-fed and immunized (n = 2)
Adipose	2.15 ± 0.16	2.90 ± 0.64	3.07 ± 1.04	5.44 ± 0.73
Adrenal	0.551 ± 0.155	0.615 ± 0.330	0.181 ± 0.057	0.153 ± 0.034
Aorta	0.008 ± 0.001	0.009 ± 0.003	0.104 ± 0.021	0.051 ± 0.015
Bone marrow	8.70 ± 1.35	10.42 ± 1.29	6.73 ± 0.35	6.03 ± 2.21
Intestine	2.18 ± 0.18	2.17 ± 0.38	2.71 ± 1.18	2.87 ± 0.04
Kidney	1.44 ± 0.21	1.58 ± 0.08	1.34 ± 0.11	1.41 ± 0.38
Liver	58.37 ± 2.33	58.22 ± 3.27	45.19 ± 0.03	39.85 ± 10.30
Lung	0.87 ± 0.19	0.79 ± 0.07	1.05 ± 0.55	1.16 ± 0.71
Skeletal muscle	3.09 ± 0.99	4.11 ± 2.01	11.57 ± 0.89	14.04 ± 13.76
Skin	5.54 ± 2.81	3.56 ± 0.72	9.00 ± 2.68	11.28 ± 2.86
Spleen	0.66 ± 0.02	0.58 ± 0.10	0.74 ± 0.44	0.86 ± 0.20
Urine	15.82 ± 1.63	14.14 ± 4.76	16.44 ± 2.01	15.16 ± 2.04
Plasma FCR (pool/hr)	0.070 ± 0.007	0.076 ± 0.003	0.032 ± 0.001	0.031 ± 0.003

At the end of each experiment all of the organs noted were sampled or collected as described in Methods. The total counts recovered in each organ are expressed as a percent of the total recovered counts (excluding residual plasma counts). Counts found in the gut contents were assumed to represent hepatic-derived counts excreted into bile and were included in the counts noted as liver. The number in parentheses refers to the number of animals studied under these conditions.

TABLE 3. Sites of degradation of glcLDL in control and cholesterol-fed rabbits. Percent contribution of individual organs to overall degradation

	Chow-Fed			Cholesterol-Fed
	A Native LDL (n = 2)	B glcLDL (5%) (n = 3)	C glcLDL (50%) (n = 2)	D glcLDL (5%) (n = 2)
Adipose	2.15 ± 0.16	2.35 ± 0.48	3.011 ± 0.54	3.56 ± 0.88
Adrenal	0.551 ± 0.155	0.601 ± 0.139	0.089 ± 0.017	0.160 ± 0.047
Aorta	0.008 ± 0.001	0.010 ± 0.001	0.031 ± 0.003	0.114 ± 0.008
Bone marrow	8.70 ± 1.35	7.48 ± 1.05	11.21 ± 6.25	7.08 ± 1.86
Intestine	2.18 ± 0.18	2.66 ± 0.04	6.10 ± 1.92	3.34 ± 0.29
Kidney	1.44 ± 0.21	1.32 ± 0.07	3.25 ± 0.75	1.52 ± 0.26
Liver	58.37 ± 2.33	57.99 ± 2.19	37.66 ± 0.93	42.20 ± 0.05
Lung	0.87 ± 0.19	0.92 ± 0.23	2.63 ± 2.02	1.19 ± 0.57
Skeletal muscle	3.09 ± 0.99	3.75 ± 1.21	4.45 ± 1.38	12.95 ± 0.98
Skin	5.54 ± 2.81	7.26 ± 3.56	12.81 ± 8.52	9.58 ± 5.52
Spleen	0.66 ± 0.02	0.61 ± 0.02	0.61 ± 0.16	0.78 ± 0.55
Urine	15.82 ± 1.63	14.34 ± 1.44	16.52 ± 1.63	15.77 ± 2.11
Plasma FCR (pool/hr)	0.070 ± 0.007	0.054 ± 0.010	0.013 ± 0.001	0.028 ± 0.004

The percent degradation of individual organs was determined as described in Methods and in the legend for Table 2. Data for column A were presented in Table 2 and are presented again to facilitate comparisons.

found in the lung was minimal. This suggests that these large immune complexes had been redistributed elsewhere. In the animal studied at 24 hr, bone marrow was greatly enriched but the liver was still the predominant site of accumulation of label (Table 4, Column C).

We previously observed that in normal chow-fed rabbits almost 90% of hepatic degradation of LDL occurred in hepatocytes (19). One might expect that in contrast, the rapid immune-mediated clearance of glcLDL would lead to nonparenchymal cell uptake of glcLDL–immune complexes. To examine this, autoradiography of the liver was performed **(Fig. 4)**. Radioactive grains were primarily concentrated in sinusoidal cells, i.e., Kupffer cells and possibly endothelial cells. However, small numbers of grains were also consistently found over parenchymal cells.

In the experiments above, glcLDL was used that had up to 50% of the lysine residues derivatized. Thus there were multiple sites for antibody attachment and large or even insoluble complexes could be formed. Since we have found that only small degrees of glucosylation of LDL occurs in vivo in diabetic patients, i.e., only 2–5% of lysine

TABLE 4. Sites of degradation of glcLDL in immunized rabbits. Percent contribution of individual organs to overall LDL degradation

	Chow-Fed				Cholesterol-Fed
	A	B	C	D	E
	Native	glcLDL (50%) (3 hr)	glcLDL (50%) (24 hr)	glcLDL (5%)	glcLDL (5%)
	(n = 3)	(n = 1)	(n = 1)	(n = 3)	(n = 2)
Adipose	2.90 ± 0.64	1.41		1.44 ± 0.42	1.34 ± 0.88
Adrenal	0.615 ± 0.330	0.013	0.007	0.087 ± 0.043	0.011 ± 0.010
Aorta	0.009 ± 0.003	0.003	0.004	0.005 ± 0.002	0.005 ± 0.002
Bone marrow	10.42 ± 1.29	8.92	26.09	2.35 ± 0.52	2.56 ± 2.54
Intestine	2.17 ± 0.38	1.65	0.42	0.92 ± 0.27	0.45 ± 0.17
Kidney	1.58 ± 0.08	0.44	0.42	0.96 ± 0.07	0.54 ± 0.13
Liver	58.22 ± 3.27	66.64	43.17	71.98 ± 4.10	72.55 ± 8.63
Lung	0.79 ± 0.07	14.87	0.64	0.28 ± 0.04	0.39 ± 0.33
Skeletal muscle	4.11 ± 2.01	1.58	0.72	1.74 ± 1.82	2.28 ± 2.36
Skin	3.56 ± 0.72	1.06	1.19	2.17 ± 0.36	1.60 ± 0.24
Spleen	0.58 ± 0.10	2.52	2.42	0.18 ± 0.06	0.34 ± 0.31
Urine	14.14 ± 4.76	0.26	24.70	17.43 ± 6.06	17.51 ± 1.54
FCR (pool/hr)	0.076 ± 0.003	10.03	3.56	0.153 ± 0.033	0.299 ± 0.198

The percent contribution of individual organs to degradation of glcLDL in immunized rabbits was determined as outlined in the legend to Table 2. In column A are shown data for three immunized animals in which native LDL was injected. In columns B and C are shown sites of degradation for a glcLDL(50%) preparation injected into immunized animals that were killed after either 3 hr or 24 hr, respectively. Column D lists sites of degradation of a glcLDL(5%) preparation. In column E are shown sites of degradation of a glcLDL(5%) preparation in two cholesterol-fed and immunized animals.

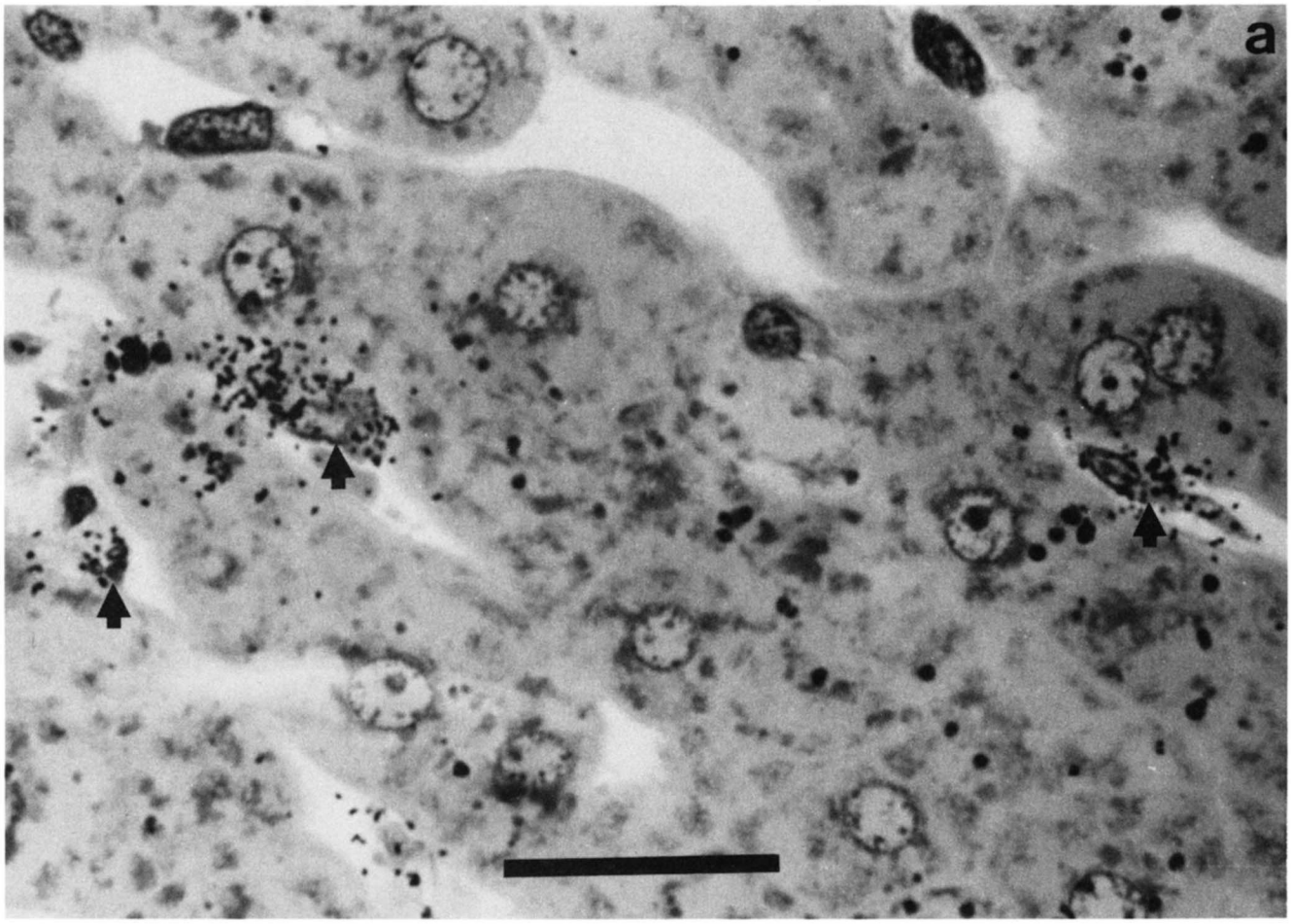


Fig. 4. Autoradiograph of a liver specimen obtained 3 hr after injection of ^{125}I-labeled TC-glcLDL(50%) into a rabbit immunized against glcLDL. In panel a is shown a bright field micrograph of a 1-micron toluidine blue-stained section. Panel b is a dark field image of the same section. Autoradiographic grains appear as dark spots in panel a and white spots in panel b. Dense clusters of grains are associated exclusively with nonparenchymal cells, indicated by arrows in panel a. Methods for tissue fixation and preparation of autoradiograms were previously described (20). Scale bar equals 20-microns.

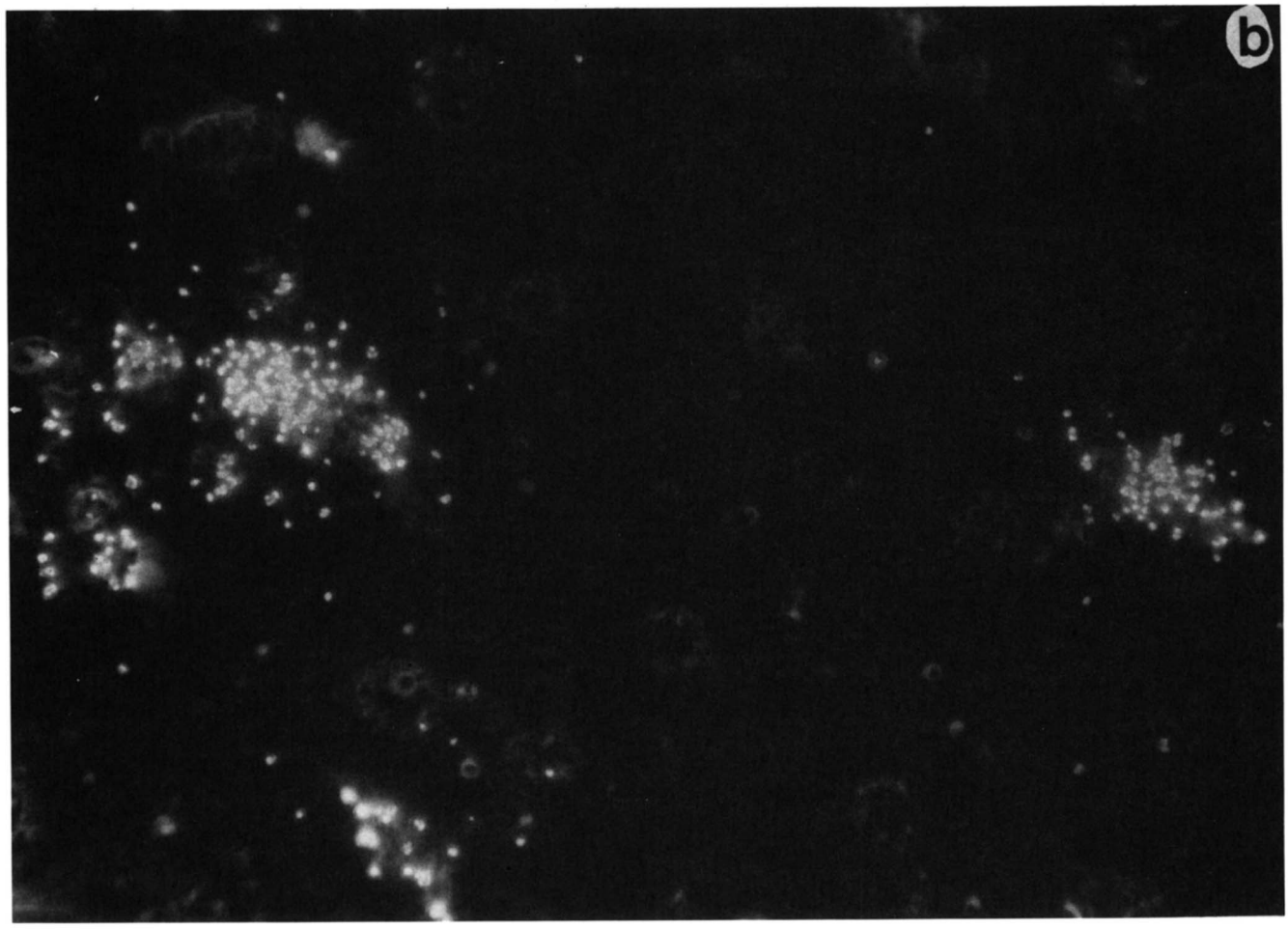
residues derivatized, we next sought to determine whether even these small degrees of modification of LDL (glcLDL(5%)) would also result in altered sites of degradation in immunized rabbits.

As noted previously, and shown in Fig. 3, the plasma clearance of glcLDL(5%) was enhanced two- to fourfold in all immunized rabbits. In the chow-fed animals, the sites of degradation of glcLDL(5%) were also significantly altered (Table 4, column D) so that the vast majority of glcLDL(5%) was targeted to the liver. Almost all other tissues showed a corresponding decrease in their contribution to overall degradation. In particular, bone marrow and spleen were markedly reduced. Uptake by adrenals was strikingly curtailed. Since adrenal uptake of glcLDL(5%) was not inhibited in nonimmune rabbits (Table 3, column B), these data suggest that the binding of antibodies to glcLDL(5%) inhibited the ability of glcLDL(5%) to interact with the adrenal LDL receptor.

Finally, we examined the impact of cholesterol feeding on the sites of degradation of glcLDL(5%) in immunized animals (Table 4, column E). Hepatic uptake was still greatly enhanced reinforcing the notion that reticuloendothelial cell uptake accounted for the hepatic clearance. Other sites of uptake were similar to that seen in the absence of cholesterol feeding. Thus, the redistribution of sites of degradation of glcLDL brought about by the presence of antibodies to glcLDL was not influenced by cholesterol feeding.

Effect of perturbations on LDL receptor pathways

Thus far the data for tissue sites of degradation have been expressed as the contribution of individual organs to the overall degradation. This is heavily dependent on the mass of each organ, i.e., skeletal muscle is actually very inactive on a per mg basis in LDL uptake but, because of its large mass, it still contributes significantly to the overall LDL clearance. In order to take into account the large difference in fractional clearance rates and in order to express data in terms of degradation per gram of tissue, the data have also been calculated as fractional clearance



rate per gram of tissue per hour. This gives an estimate of the portion of the plasma pool that is cleared per gram of each individual tissue per hour. This calculation makes the assumption that a true steady state can occur, and that glcLDL was continuously forming and complexing with immunoglobulin. Data for adrenal and liver, the most active tissues, are given in **Fig. 5**, and data for spleen, bone marrow, and ovaries are given in **Fig. 6**.

In agreement with previous reports (25, 26), the adrenals were the most active in degradation of native LDL (Fig. 5). This uptake was equally inhibited by down-regulation of LDL receptors achieved by cholesterol feeding (bar E) or by blocking LDL's ability to interact with its receptor achieved by extensive glucosylation (bar C). Since most LDL uptake in adrenals is receptor-dependent, this is in good agreement with earlier observations (19). Our data also show that cholesterol feeding inhibits *both* hepatic (25, 26) and extrahepatic LDL receptor-mediated cellular uptake. Our data also demonstrate that glucosylation of LDL blocks the receptor binding of LDL in vivo (22). Glucosylation of only 5% of lysine residues did not impair adrenal uptake (bar B, Fig. 5) as judged by this technique, but the presence of

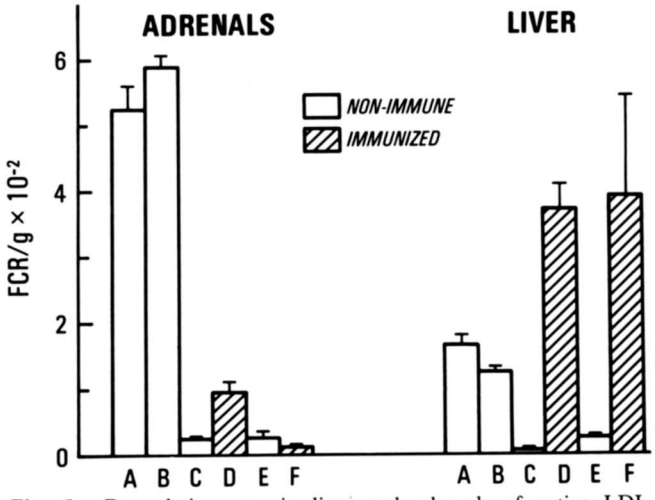


Fig. 5. Degradation rate in liver and adrenals of native LDL, glcLDL(5%), and glcLDL(50%) in control rabbits, cholesterol-fed rabbits, and in rabbits immunized against glcLDL. Data are expressed as the percentage of the total plasma pool degraded per gram of tissue (FCR/g) (see text for rationale for presenting data in this fashion). Bar a, control rabbits injected with native LDL; bar b, control rabbits injected with glcLDL(5%); bar c, control rabbits injected with glcLDL (50%); bar d, immunized rabbits injected with glcLDL (5%); bar e, nonimmunized, cholesterol-fed rabbits injected with native LDL; bar f, immunized and cholesterol-fed rabbits injected with glcLDL(5%).

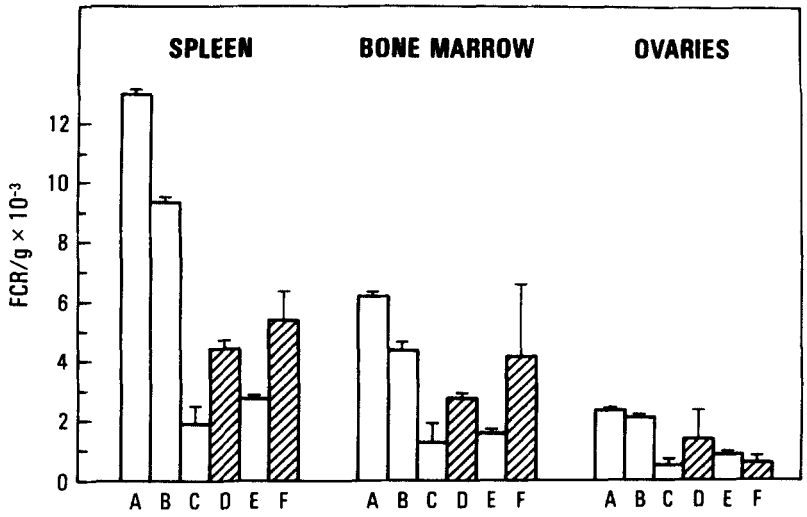


Fig. 6. Degradation rate in spleen, bone marrow, and ovaries of native LDL, glcLDL(5%), and glcLDL(50%) in control rabbits, cholesterol-fed rabbits, and in rabbits immunized against glcLDL. Data are expressed as FCR/g (see text for discussion). Bar a, control rabbits injected with native LDL; bar b, control rabbits injected with glcLDL(5%); bar c, control rabbits injected with glcLDL(50%); bar d, immunized rabbits injected with glcLDL(5%); bar e, nonimmunized, cholesterol-fed rabbits injected with native LDL; bar f, immunized and cholesterol-fed rabbits injected with glcLDL(5%).

humoral antibodies to glcLDL significantly inhibited the ability of glcLDL(5%) to interact with the LDL receptor (bar D). The inhibition of adrenal uptake of glcLDL(5%) in the immunized animals was augmented by cholesterol feeding (bar F). In nonimmunized animals, qualitatively similar results were seen in the liver. However, when immune-mediated clearance took place, the overall hepatic uptake was greatly enhanced. This is consistent with the non-LDL receptor, non-parenchymal cell uptake shown in Fig. 4.

Results similar to those seen in the adrenals were also found in the ovaries (Fig. 6). Uptake of glcLDL(5%) in the spleen and bone marrow of the immunized animals was similar to that seen in the liver, consistent with an immune clearance mediated by the reticuloendothelial cells of these organs.

Uptake of native and glcLDL in aorta

The overall contribution of aorta to the degradation of LDL is small. However, even a small increase in LDL uptake, when integrated over a long period of time, could have an important impact on cholesterol deposition in the arterial wall.

With the present experimental technique, the percent contribution of the aorta to total LDL uptake in the control rabbits was similar for native LDL and for glcLDL(5%). For glcLDL(50%) a higher uptake was observed (Table 3). Cholesterol feeding also caused a substantial increase in aortic uptake of LDL and glcLDL(5%) (Table 2 and Table 3). This increase was more pronounced in the more atherosclerotic thoracic aorta than in the less affected abdominal aorta (data not shown). Whether the higher uptake in atherosclerotic tissue reflects extracellular deposition or intracellular degradation, or both, could not be concluded from these experiments.

In *immune* rabbits, in *all* experimental situations, the aortic contribution to uptake of glcLDL was *lower* than that with native LDL. This was true even in the immune, cholesterol-fed animals, i.e., the glcLDL-immune complexes were being redirected to the liver.

DISCUSSION

An animal model was developed to study LDL degradation under different conditions, especially in the presence of autologous antibodies against glcLDL. In earlier studies we investigated the sites of degradation of LDL in normal rabbits and in LDL receptor-deficient rabbits (19). In the previous studies [^{14}C]sucrose was used as a "trapped label" tracer, while in the present study ^{125}I-labeled TC was used. The data on sites of LDL degradation in control rabbits in the present study conform closely to earlier obtained data, with the main mass of LDL degraded by the liver. The adrenals were found to be the most active organ in LDL degradation per gram tissue. In the present study specimens were also obtained from the bone marrow, which was not done earlier. These data show that even in normal animals the bone marrow is one of the main LDL-degrading organs (Table 3).

Comparing our data previously obtained in the LDL receptor-deficient rabbits (19) with the data presented here for cholesterol-fed rabbits, it can be concluded in this

study conducted with a limited number of animals that cholesterol feeding produces changes in the plasma decay of LDL and in LDL degradation sites very similar to those observed in receptor-deficient animals. Thus in the cholesterol-fed rabbits, the FCR for LDL is reduced to 30–50% of that in chow-fed rabbits, and the degradation rate per gram of tissue is dramatically reduced in liver, adrenals, and ovaries. These results demonstrate that cholesterol feeding induces a down-regulation of *both* hepatic and nonhepatic LDL receptors. The rate and sites of degradation of glcLDL(50%) in normal animals are also very similar to those observed for native LDL in cholesterol-fed animals, but in this case, of course, the *LDL* is unable to react with its receptors.

An important aim of this study was to investigate how the presence of autologous antibodies affected the catabolic rate of LDL and its sites of degradation. The significance of autoantibodies against lipoproteins was first suggested by Beaumont and Beaumont in so-called "autoimmune hyperlipidemia" (1). Subsequently, autoantibodies against LDL were demonstrated by several investigators in states with paraproteinemia, as well as in other conditions (2–8). The presence of autoantibodies against LDL has in most cases been associated with hyperlipidemia, but also with hypolipidemia (27). Recently a patient was reported who apparently had an autoantibody against the LDL receptor, resulting in hypercholesterolemia (28). In some of these reports deposition of immunoglobulins and lipid has been found in various tissues, suggesting a deposition of immune-complexes (2, 4). In these conditions, deposition of such immune-complexes in the arterial wall could be a mechanism for cholesterol deposition favoring the development of atherosclerosis. The possibility of such a mechanism is supported by the presence of IgG in atherosclerotic plaques (9, 10). The increased frequency of circulating immune-complexes in disorders related to atherosclerosis has further supported the idea that immunological mechanisms may play a role in the development of the atherosclerotic plaque (11–14). Such mechanisms are also supported by observations in animal studies showing immune-complex depositions in the vessel wall (29) as well as a synergistic effect of allergic injury and cholesterol feeding on the development of atherosclerosis in rabbits (30, 31).

In the present study, LDL was made immunogenic by modification by reductive glucosylation in order to obtain circulating antibodies against the modified LDL. Since glucosylated LDL as well as antibodies against reductively glucosylated LDL have been shown in humans (18), similar mechanisms may be operating in vivo. Furthermore several subtle modifications that theoretically may occur in vivo have been shown to make LDL immunogenic in animal models (16).

In this study, a subtle modification of LDL (glcLDL(5%)) was found to inhibit the clearance rate of LDL in control rabbits; in contrast, in the immunized animals the rate of clearance was accelerated 2-fold, suggesting that even this minor degree of modification yielded a product that was recognized as an antigen in the immunized rabbits. With a higher degree of modification the clearance rate was increased 50- to 100-fold. Similar results have been reported when preformed immune complexes of heterologous LDL (human LDL), and rabbit antibodies were injected into rabbits (32).

When compared to native LDL, the increased degradation of glcLDL in the immunized rabbits was accounted for primarily by the liver, and primarily by reticuloendothelial cells of the liver. In the experiments in which glcLDL(50%) was injected, increased deposition was also noted in the lung and bone marrow. Presumably, this represents reticuloendothelial cell trapping and clearance of large immune complexes which formed when multiple antibodies bound to the heavily derivatized LDL (there would be about 160 mol of glucitollysine per mole LDL to which antibodies could bind). In addition, the antibodies appeared to alter the normal sites of LDL degradation in still another manner. In the *immunized* animals (but not normal animals) there was a decreased degradation rate of glcLDL(5%) in organs rich in LDL receptors, e.g., the adrenals. This suggests that the immunoglobulin bound to glcLDL inhibited recognition of LDL by the LDL receptor, presumably by steric interference. In turn, the reticuloendothelial cells very efficiently removed these complexes. Thus, these studies in vivo are consistent with in vitro cell culture studies in which the presence of anti-LDL antibodies interfered with LDL receptor-binding (5, 32, 33).

Under the experimental conditions used in the present study there was no evidence for specific targeting of LDL–immunoglobulin complexes to the aortic wall, rather they were targeted primarily to the liver. In a similar manner, any immunogenic modification of LDL (16), perhaps such as might occur with peroxidation of LDL, would presumably lead to targeting of the modified LDL to the liver. However, if modifications of LDL occurred in vivo at a steady rate, and then the modified LDL complexed with existing antibodies, such that the overall fractional removal from plasma was greatly accelerated, then the overall, absolute deposition in the artery could be increased. Whether or not this actually occurs is unknown.

Since many of the foam cells in the developing atherosclerotic lesions are monocyte-derived macrophages (34, 35), these cells could be assumed to actively take up and accumulate immune complexes via their Fc-receptors. It has also recently been reported that macrophages in cell culture may take up and accumulate

lipid from LDL-containing immune complexes (32). To enrich the arterial intima with macrophages, we induced atherosclerosis in rabbits by cholesterol feeding, and used these animals to study the sites of degradation of LDL-immune-complexes. Even in this situation there was no evidence for enhanced uptake of glcLDL in the aorta in immunized rabbits as compared to controls. This suggests that even in atherosclerotic animals the hepatic reticuloendothelial system removes the immune-complexes efficiently, presumably preventing their uptake by arterial wall macrophages.

However, the studies in the cholesterol-fed animals did demonstrate a dramatically increased uptake of LDL by the arterial wall. This might be due to an increased influx of LDL into the atherosclerotic tissue leading to extracellular deposition of intact LDL, as well as to an increased catabolism of LDL in the more cellular intima of the atherosclerotic vessel. The contribution of these mechanisms cannot be concluded from the present data but are currently under study.

In conclusion, we have presented an animal model for studies of the metabolic effects of autoantibodies against LDL. These effects, as found in the present study, can be summarized as an overall increased rate of removal from plasma of the modified LDL, reflecting an efficient uptake and degradation by the hepatic reticuloendothelial system, while the LDL receptor-dependent uptake is reduced. Under the present experimental conditions, no targeted deposition of immune-complexes in the aortic wall could be shown.

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