Enhanced Hepatic Uptake and Processing of Cholesterol Esters From Low Density Lipoprotein by Specific Lactosaminated Fab Fragments

Martin K. Bijsterbosch, Franco Bernini, Hille F. Bakkeren, Antonio M. Gotto Jr., Louis C. Smith, and Theo J.C. van Berkel

Reduction of the blood levels of low density lipoprotein (LDL) is important for lowering the incidence of atherosclerosis. In this study, LDL was directed to rat parenchymal liver cells by lactosaminated Fab fragments of anti-apolipoprotein B antibodies (LacFab). We followed the fate of intravenously injected complexes of LacFab and $[^3H]$cholesteryl oleate-labeled LDL. Complexing of LacFab to LDL led to rapid disappearance of LDL from the circulation. At 30 minutes after injection, the liver contained 58.5 ± 9.0% of the injected dose (at that time the liver contained only 5.7 ± 2.2% of an injected dose of free LDL). Liver uptake was blocked by N-acetylgalactosamine but not by N-acetylglucosamine, which indicates that galactose-specific recognition sites are responsible for the LacFab-induced hepatic uptake. By isolating liver cells, it was found that parenchymal, endothelial, and Kupffer cells account for 87%, 3%, and 10% of the total hepatic uptake, respectively. Subcellular fractionation of the liver indicated that the complexes are rapidly internalized and transported to lysosomes. Within 1 hour after injection, virtually all the $[^3H]$cholesteryl oleate of the internalized LDL was hydrolyzed; hydrolysis was followed by excretion of radioactivity into the bile. Compared with rats injected with native $[^3H]$cholesteryl oleate-labeled LDL, eight times as much radioactivity was excreted into the bile during the first 4 hours after the injection of LacFab-complexed $[^3H]$cholesteryl oleate-labeled LDL. Thus, LacFab induces enhanced hepatic uptake of LDL via galactose receptors on the parenchymal cells, followed by processing in lysosomes and excretion into the bile. In this way, LacFab induces an increased irreversible removal of LDL cholesterol from the body. (Arteriosclerosis and Thrombosis 1991;11:1806–1813)

The level of low density lipoprotein (LDL) in plasma depends on the presence and activity of specific hepatic LDL (apolipoprotein [apo] B, E) receptors.1–3 If the normal handling of LDL via these receptors is impaired as a consequence of defects in either the receptor or the apolipoprotein, the levels of LDL in the circulation are increased, which is correlated with an increased risk of atherosclerosis.3–5 Hypercholesterolemia often be treated with inhibitors of hydroxymethylglutaryl-coenzyme A reductase (the rate-limiting enzyme in the synthesis of cholesterol) and/or bile acid sequestrants.3,6 However, patients with receptor-negative familial hypercholesterolemia (FH) do not respond appreciably to these drugs.7 Furthermore, in patients with familial defective apo B-1008 these drugs may be of limited value.9

We explored the possibility of reducing LDL levels by removing circulating LDL via alternative receptor mechanisms, in particular hepatic galactose receptors. The liver contains two separate receptor systems that specifically bind and internalize D-galactose-terminated compounds.10,11 The parenchymal cells express the asialoglycoprotein receptor,10 whereas Kupffer cells contain the so-called "galactose-particle receptor."11–13 The two receptor systems have the same carbohydrate specificity, but they differ in their capacity for uptake of variously sized ligands. Kupffer cells internalize galactose-terminated particles with a
diameter of >10–15 nm. These cells are, however, unable to take up small galactose-terminated ligands. Parenchymal cells, on the other hand, are well known for their rapid uptake of small galactose-terminated ligands such as glycoproteins. It has, however, been suggested that uptake of galactose-terminated particles by these cells is limited to particles with a diameter of <10–15 nm.13

We have shown earlier that the incorporation of a galactose-cholesterol derivative (tris-gal-chol) into LDL induces rapid galactose-specific uptake by the liver. The hepatic uptake was due mainly to Kupffer cells, whereas the contribution of parenchymal cells was very low.14 However, tris-gal-chol is not specific for LDL, and it also induces the uptake of high density lipoprotein (HDL). Recently, a more LDL-specific reagent was developed: lactosaminated Fab fragments of anti-LDL antibodies (LacFab). Intravenously injected LacFab formed immune complexes with circulating LDL, which subsequently associated with the liver. This resulted in a substantial and specific reduction of the LDL level in the plasma.16,17 Histological studies with fluorescently labeled LDL gave the impression that LacFab-LDL associated with the parenchymal cells, which was at variance with the claim that these cells are unable to bind such a large complex (the diameter of LDL is 23 nm).13 Because internalization by parenchymal cells may be coupled directly to biliary excretion, uptake of LacFab by these cells will afford a very promising therapeutic perspective. It is therefore crucial to determine the site of hepatic uptake of the complex, and so we quantitatively assessed the contributions of different cell types to the association of LacFab with the liver.

The coupling of a large particle such as LDL to the much smaller LacFab may affect the intracellular destination of the latter (the lysosomal compartment). It has been shown, for instance, that coupling of even small gold particles to transferrin dramatically alters the intracellular routing of this ligand.18 For the proper handling of LacFab-LDL, it is essential that the complex be internalized and transported to lysosomes. We therefore studied the hepatic processing of LacFab-LDL, and to establish the fate of the cholesterol moiety of LDL, we used complexes of LacFab and [3H]cholesterol olate-labeled LDL. We also studied the excretion of radioactivity into the bile because for the irreversible disposal of LDL cholesterol (esters) from the body, uptake of LacFab-LDL by the liver should be coupled to biliary excretion.

Methods

Reagents

1-[\alpha,\beta\alpha(n)-3H]cholesterol olate was supplied by Amersham International, Amersham, UK. Collagenase type I, N-acetyl-d-galactosamine, N-acetyl-p-glucosamine, bovine serum albumin (fraction V), agarose-bound neuraminidase (from Clostridium perfringens; type VI-A), and fetuin (type IV) were obtained from Sigma Chemical Co., St. Louis, Mo. Soluene-350 and the scintillation cocktails Emulsifier Safe and Hionic Fluor were from Packard Instruments Co., Downers Grove, Ill. All other chemicals were of analytical grade.

Fetuin was desialylated enzymatically by incubating the protein, dissolved in 0.1 M sodium acetate buffer, pH 5.5, with 20 milliunits/ml agarose-bound neuraminidase for 4 hours at 37°C. A minimum of 80% of the sialic acid residues, assayed as described earlier,19 was removed by this procedure.

Lactosaminated Fab fragments of polyclonal anti-human LDL antibodies (LacFab) were prepared as described previously.16

Isolation and Radiolabeling of Low Density Lipoprotein, Formation of Immune Complexes

Human LDL (density 1.019–1.063 g/ml) was isolated by two repetitive centrifugations as described earlier.20 The lipoprotein was radiolabeled by the incorporation of [3H]cholesterol olate. The incorporation was carried out essentially as described by Blomhoff et al,21 using the cholesteryl ester transfer protein present in human serum. Twenty-five microcuries of [3H]cholesterol olate, dissolved in 100 μl acetone, was mixed with 1 ml lipoprotein-deficient human serum (obtained by density gradient centrifugation; density of >1.21 g/ml), and the acetone was evaporated under a stream of nitrogen. After 20 minutes at room temperature, the mixture was shaken for 10 minutes at 37°C. Then 1 ml of an LDL solution (1–2 mg apolipoprotein/ml in phosphate-buffered saline) was added, and the mixture was incubated for 5 hours at 37°C. Immune complexes of LacFab and LDL were subsequently formed by the addition of antibody at a 25–50 molar excess. After 1 hour of incubation at room temperature, the immune complexes were purified by density gradient centrifugation.20 By measuring the protein to cholesterol mass ratio of the complexes and assuming molecular weights for LacFab and apo B of 46,000 and 512,000, respectively, it was calculated that approximately 10 molecules of LacFab were associated with each LDL particle. The specific radioactivity was 8–14×10⁶ dpm/mg protein.

In Vivo Serum Clearance and Liver Association

Male Wistar rats weighing approximately 300 g were used. The animals were anesthetized by the intraperitoneal injection of 15–20 mg sodium pento-barbital. The abdomen was opened, and radiolabeled immune complexes were injected into the inferior vena cava. At the indicated times, blood samples of 0.2–0.3 ml were drawn from the inferior vena cava. The samples were centrifuged for 2 minutes at 16,000g. Duplicate samples of serum were assayed for radioactivity. The total amount of radioactivity in the serum was calculated using the equation

\[
\text{Serum volume in milliliters} = \left(0.0219 \times \text{body weight in grams}\right) + 2.66
\]
(determined by injecting rats with radioiodinated serum albumin; see Reference 22).

At the indicated times liver lobules were tied off and excised, and at the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successively did not exceed 15% of the total liver mass. Radioactivity in the liver at each time was calculated from the radioactivities and weights of the liver samples and corrected for the radioactivity of the plasma assumed to be present in the tissue at the time of sampling (85 μL/g fresh wt; see Reference 23).

Isolation of Liver Cells

Rats were anesthetized and injected with radiolabeled immune complexes as described in the previous section. Ten minutes later, the vena portae was cannulated and the liver was perfused with Ca²⁺-free Hanks' balanced salt solution containing 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.4, at 8°C and a flow rate of 14 ml/min. After 8 minutes, a lobule was tied off for determination of the total liver uptake. Then the liver was perfused with 0.05% (wt:vol) collagenase in Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4, and parenchymal and nonparenchymal cells were isolated as described previously. The nonparenchymal cell preparation was further fractionated into endothelial and Kupffer cells by centrifugal elutriation as described in detail earlier. Contributions of the various cell types to the total liver uptake were calculated as described previously. As found earlier with other substrates, no significant amounts of radioactivity were lost from the cells during the isolation procedure. This was checked in each experiment by comparing the calculated liver uptake (i.e., the sum of the contributions of the various cell types) with the value actually measured in the liver lobule.

Bile Collection

Male Wistar rats were equipped with permanent catheters in the bile duct, the duodenum, and the heart as described in detail earlier. The enterohepatic circulation was preserved by connecting the bile duct and duodenum catheters. The rats were allowed to recover from the surgery for 1 week before they were used for experiments. The animals were not anesthetized during the experiments, and they had free access to food and water. Radiolabeled LDL and LacFab-LDL immune complexes were digested with Soluene-350 and, if necessary, bleached with 30% hydrogen peroxide. Bile samples were bleached by the addition of an equal volume of 30% hydrogen peroxide. Samples of plasma and labeled protein were counted without further processing. Radioactivity was measured by liquid scintillation counting, using Emulsifier Safe or Hionic Fluor scintillation cocktails.

Determination of Proteins

Protein concentrations in homogenates, cell suspensions, and solutions of LDL and antibody were determined by the method of Lowry et al, with bovine serum albumin as the standard. The values found for LDL were multiplied by a factor 0.82 to correct for the higher color yield of apo B.

Results

Plasma Clearance and Liver Uptake of LacFab–Low Density Lipoprotein Immune Complexes

Native LDL disappears slowly from the circulation of the rat (half-life of >4 hours). At 10 minutes after the intravenous injection of [³H]cholesteryl oleate–labeled native LDL into rats, only 5.7±2.2% (mean±SEM; three animals) of the injected dose was found in the liver. In sharp contrast, complexes of LacFab with [³H]cholesteryl oleate–labeled LDL were cleared very rapidly from rat plasma (Figure 1). At 30 minutes after the injection, only 29.8±2.4% of the injected dose was left in the plasma. At that time the liver contained 58.5±9.0% of the injected dose, which represents 84% of the cleared radioactivity.

To determine the mechanism of the liver association, rats were preinjected with either N-acetylgalac-
tosamine or N-acetylglucosamine 1 minute before injection of the labeled complexes. At 10 minutes after injection the association of radioactivity with the liver was determined. Table 1 shows that preinjection with 400 mg/kg body wt N-acetylgalactosamine almost completely inhibited the association of LacFab-LDL with the liver. Preinjection with the same dose of N-acetylglucosamine had no effect. These findings indicate that galactose-specific recognition sites are responsible for the hepatic association of the immune complexes.

**Table 1. Effects of N-Acetylgalactosamine and N-Acetylglucosamine on Liver Uptake of LacFab-LDL in Rats**

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Liver association (% of dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate-buffered saline</td>
<td>61.5±6.0</td>
</tr>
<tr>
<td>N-Acetylgalactosamine</td>
<td>5.7±1.4*</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>62.2±10.8</td>
</tr>
</tbody>
</table>

LacFab-LDL, lactosaminated Fab fragments complexed to low density lipoprotein. Values are mean±SEM of three or four rats. *p<0.05 different from saline controls by Wilcoxon's two-sample test.

**Figure 2. Line plot of hepatic processing of immune complexes of lactosaminated Fab fragments of anti-apolipoprotein B antibodies (LacFab) and \[^3H\]cholesterol oleate-labeled low density lipoprotein (LDL).** Rats were injected with immune complexes of LacFab and \[^3H\]cholesterol oleate-labeled LDL (50 \(\mu\)g apolipoprotein/kg body wt). At indicated times, total liver radioactivity (o) and amounts of \[^3H\]cholesterol oleate (c) and \[^3H\]cholesterol (e) in liver were determined. Values are mean±SEM of three rats.

Asialofetuin blocks uptake via the galactose-specific receptors on parenchymal liver cells, but not galactose-mediated uptake by Kupffer cells.32 In animals preinjected with 50 \(\mu\)g/kg body wt asialofetuin, only 5.4±1.6\% of the dose was found in the liver at 10 minutes after the injection of radiolabeled LacFab-LDL (mean±SEM of three rats; livers of control rats contained 61.5±6.0\% of the dose). This finding emphasizes the important role of parenchymal cells in the association of the immune complexes with the liver.

**Hepatic Processing of LacFab-Low Density Lipoprotein**

Processing of LacFab-LDL by the liver was followed by measuring the amounts of \[^3H\]cholesterol oleate and \[^3H\]cholesterol in the liver at various times after the injection of immune complexes of LacFab and \[^3H\]cholesterol oleate-labeled LDL. Figure 2 shows that shortly after injection most of the radioactivity in the liver was in \[^3H\]cholesterol oleate. Between 10 and 30 minutes after injection, the amount of \[^3H\]cholesterol oleate in the liver decreased substantially. The amount of \[^3H\]cholesterol increased concomitantly, which indicates that the cholesterol esters of LDL are rapidly hydrolyzed by esterases to free cholesterol. The cholesterol may then become available for further processing such as the synthesis of bile acids. The accumulation of such subsequent radioactive metabolites was not measured, which explains the incomplete recovery of the total radioactivity at this point.

The intracellular processing of the complexes was determined by subcellular fractionation of the liver at 30 minutes after the injection of \[^3H\]cholesterol oleate-labeled LacFab-LDL. Figure 3 shows that the distribution pattern of radioactivity closely resembled...
FIGURE 3. Distribution patterns of radioactivity and marker enzymes over subcellular fractions of liver after injection of immune complexes of lactosaminated Fab fragments of anti-apolipoprotein B antibodies (LacFab) and \(^{3}H\)cholesteryl oleate-labeled low density lipoprotein (LDL). Rats were injected with immune complexes of LacFab and \(^{3}H\)cholesteryl oleate-labeled LDL (50 \(\mu\)g apolipoprotein/kg body wt). Thirty minutes after injection, liver was perfused with ice-cold 0.25 M sucrose containing 10 mM Tris-HCl buffer, pH 7.5, and divided into subcellular fractions by differential centrifugation as described earlier. Fractions were assayed for radioactivity, protein, and activity of several marker enzymes; recoveries were 76–128%. Blocks from left to right represent nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P), and supernatant (cytosol, S) fractions. Relative protein concentration is given on the y axis. X axis represents relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein). Tris, tris(hydroxymethyl)aminomethane.

that of the lysosomal marker acid phosphatase, whereas the microsomal marker glucose-6-phosphatase and the cytoplasmic marker lactate dehydrogenase show clearly different distributions. This indicates that, after binding to receptors on the plasma membrane, the complexes are internalized and transported to lysosomes.

The biliary excretion of radioactivity was measured up to 12 hours after the injection of native \(^{3}H\)cholesteryl oleate-labeled LDL or immune complexes of LacFab and \(^{3}H\)cholesteryl oleate-labeled LDL (Figure 4). After the injection of native \(^{3}H\)cholesteryl oleate-labeled LDL, only 0.8±0.3% and 7.5±0.7% of the injected dose was excreted into the bile during the first 4 and 12 hours, respectively. Much more radioactivity was found in the bile after injection of the labeled LacFab-LDL complex. Compared with animals injected with labeled native LDL, eight times as much radioactivity was excreted into the bile during the first 4 hours after injection. If rats were preinjected with 50 mg/kg asialofetuin, the increase in radioactivity excreted in the bile after the injection of radiolabeled LacFab-LDL was almost completely blocked (Figure 5). Only 1.5±0.2% of the dose was found in the bile during the first 4 hours after injection. This finding indicates that the increased biliary excretion is provoked by uptake of the complex via galactose-specific receptors on parenchymal liver cells.

Discussion

We show in this study that immune complexes of LDL and LacFab are rapidly removed from the circulation by galactose-specific receptors on parenchymal liver cells. The complexes are internalized and transported to the lysosomes, and the processed products of the cholesterol esters of LDL are excreted into the bile.

Injection of LacFab into rats induces a substantial reduction in the levels of LDL, whereas HDL levels are not affected. Using radioiodinated LDL as a tracer, it was found that the injection of LacFab results in the association of LDL with the liver. Because physiologically the fate of cholesterol in LDL is important, in the present study we used LDL labeled with \(^{3}H\)cholesteryl oleate. Rats have no cholesteryl ester exchange protein in their serum.
FIGURE 4. Curves showing biliary excretion of radioactivity after injection of immune complexes of lactosaminated Fab fragments of anti-apolipoprotein B antibodies (LacFab) and [3H]cholesterol oleate-labeled low density lipoprotein (LDL). Rats were injected with native [3H]cholesterol oleate-labeled LDL (•) or with immune complexes of LacFab and [3H]-cholesterol oleate-labeled LDL (○), both at a dose of 50 μg apolipoprotein/kg body wt. Bile was collected for 12 hours and assayed for radioactivity. Results are expressed as percentage of dose per hour (panel A) and as total amount of radioactivity excreted (panel B). Values are mean±SEM of three rats.

FIGURE 5. Curves showing effect of asialofetuin on biliary excretion of radioactivity after injection of immune complexes of lactosaminated Fab fragments of anti-apolipoprotein B antibodies (LacFab) and [3H]cholesterol oleate-labeled low density lipoprotein (LDL). Rats were injected with immune complexes of LacFab and [3H]cholesterol oleate-labeled LDL (50 μg apolipoprotein/kg body wt). Animals had either not been pretreated (○) or had been preinjected with asialofetuin (△; 50 mg/kg body wt at 1 minute before injection of radiolabeled complexes). Bile was collected for 12 hours and assayed for radioactivity. Results are expressed as percentage of dose per hour (panel A) and as total amount of radioactivity excreted (panel B). Values are mean±SEM of three rats.

and the label is therefore not exchanged in the circulation but is taken up as an integral component of the LDL particle. We prepared complexes of LDL and LacFab in vitro and subsequently injected LacFab-LDL intravenously into rats. The behavior of such preformed complexes in the circulation is similar to the behavior of separately injected LDL and LacFab.17

In the liver, both Kupffer and parenchymal cells possess receptors that specifically bind and internalize D-galactose-terminated compounds.10,11 The receptor on the parenchymal cells is the classical asialoglycoprotein receptor originally described by Ashwell and Morell.10 The receptor on Kupffer cells, which is different from the receptor on parenchymal cells, was characterized more recently.11-13 It was suggested that parenchymal cells can internalize only galactose particles with a diameter of <10–15 nm and that larger particles are taken up solely by Kupffer cells.13 Indeed, we found earlier that the tris-gal-chol-induced hepatic uptake of LDL (diameter of 23 nm) occurred mainly in Kupffer cells and that parenchymal cells accounted for only <10% of the uptake.14 In sharp contrast, this study demonstrates that coupling of LacFab to LDL forms complexes that are taken up in the liver primarily by parenchymal cells. Evidence for the specific uptake by parenchymal cells was obtained from both cell separation experiments and experiments with asialofetuin, a specific competitor for galactose-mediated uptake by parenchymal cells. These present quantitative data support the impression obtained from earlier histological studies using fluorescently labeled LDL.17

Our study provides important new information about the specificities of the two galactose-specific receptor systems in the liver. The present data indicate that the mode of coupling of galactose to LDL is crucial for the relative contributions of parenchymal and Kupffer cells to hepatic uptake of the particle. It was found earlier that incorporation of tris-gal-chol into the lipid moiety of LDL induces uptake by Kupffer cells but virtually no uptake by parenchymal cells. We demonstrated recently that the direct coupling of galactose to the apoprotein of LDL results in a hepatic uptake of which only 30–60% is by parenchymal cells.35 The present study indicates, however, that if galactose is coupled to the apoprotein via a spacer (i.e., the Fab fragment), then LDL is directed specifically to parenchymal cells. We conclude, there-
efore, that a specific spatial arrangement of galactose residues on LDL is required to induce a high uptake of the particle by parenchymal liver cells and to avoid recognition by the galactose receptors on Kupffer cells.

Binding of LacFab-LDL to the galactose receptors is followed by internalization and transport to the lysosomes, which is the normal course of events for a galactose-terminated ligand such as LacFab. It is surprising that attachment of the large LDL particle to the much smaller LacFab did not affect the uptake or the intracellular routing of the latter. It has been shown, for instance, that the coupling of even small gold particles to transferrin dramatically alters the intracellular routing of this ligand.\textsuperscript{18} The lysosomes are also involved in the processing of LDL that is taken up via the LDL receptors.\textsuperscript{36,37} Thus, LacFab directly LDL via a different receptor to the same cellular compartments that are involved in the normal physiological handling of the particle. Once in the lysosomes, cholesterol esters are hydrolyzed to free cholesterol. The cholesterol then becomes available for further processing (e.g., for the synthesis of bile acids). A substantial proportion of the injected dose is excreted via the bile into the lumen of the gut. In the present study we did not determine the composition of the bile. However, we have found earlier that after induction of galactose-specific hepatic uptake of \textsuperscript{3H}cholesteryl oleate–labeled lipoproteins <10% of the biliary radioactivity was present in cholesterol and >90% in various bile acids.\textsuperscript{38}

The specific LDL-reducing properties of LacFab may have relevance for the treatment of patients with high LDL cholesterol levels. The plasma levels of LDL in patients with heterozygous FH can be reduced by bile acid sequestrants and inhibitors of hydroxymethylglutaryl–coenzyme A reductase. However, this therapy does not appreciably reduce the high LDL levels in patients suffering from homozygous FH.\textsuperscript{7} Furthermore, these drugs may be of limited value in patients with familial defective apo B-100.\textsuperscript{9} The capacity of LacFab to increase specifically the catabolism of LDL via the hepatic recognition and transport of circulating glycoproteins. \textit{Adv Enzymol} 1974;41:99–128


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