Relative roles of the LDL receptor, the LDL receptor-like protein, and hepatic lipase in chylomicron remnant removal by the liver

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Abstract Studies were carried out in mice utilizing inhibitors of several cell surface molecules to evaluate their relative roles in chylomicron remnant removal. Anti-LDL receptor antibody inhibited ~45% of rapid remnant removal from plasma, prolonged their half life (63 s to 115 s) and reduced hepatic uptake by 45%. Receptor-associated protein (RAP) (1 mg/mouse), a high affinity inhibitor of the LDL receptor-related protein (LRP) and a low affinity inhibitor of the LDL receptor decreased remnant removal ~55%, prolonged the half life from 63 s to 230 s, and reduced hepatic uptake by 70%. RAP, but not anti-LDL receptor antibody, inhibited splenic uptake. With both injected together, an incremental effect was seen; plasma removal decreased 60%, $T_{1/2}$ increased to 290 s, and hepatic uptake decreased by 80%. Thus, it is likely that virtually all of the very rapid removal of remnants from the plasma by the liver requires the presence of at least one of these members of the LDL receptor family. Anti-hepatic lipase antibody caused a small but significant delay in remnant removal from plasma and a larger decrease in hepatic uptake (22.5%). It doubled adrenal uptake. The anti-hepatic lipase antibody was not additive with either the anti-LDL receptor antibody or RAP. Anti-rat hepatic lipase antibody did not inhibit lipolysis by mouse hepatic lipase, suggesting that lipolysis is not the way hepatic lipase enhances remnant uptake. Hepatic lipase bound to remnants to a greater degree than it bound to other lipoproteins. Together these data suggest that hepatic lipase may serve as a binding site for chylomicron remnants, thereby enhancing their affinity for the liver surface, and thus removal by the proteins of the LDL receptor family. Other molecules may also play a role in removal from the circulation under conditions where the LDL receptor family receptors are absent or occupied.—**de Faria, E., L. G. Fong, M. Komaromy, and A. D. Cooper.** Relative roles of the LDL receptor, the LDL receptor-like protein, and hepatic lipase in chylomicron remnant removal by the liver. *J. Lipid Res.* 1996. **37:** 197–209.

Supplementary key words lipoprotein • chylomicron remnants • hepatic lipase • LDL receptor • liver

The precise mechanism whereby the liver rapidly and specifically removes chylomicron remnants continues to be an area of uncertainty (1). Recent genetic, physiologic, and biochemical studies have produced three general and not mutually exclusive hypotheses. The two-receptor hypothesis suggests that both the low density lipoprotein (LDL) receptor and the LDL receptor-like protein (LRP) can individually mediate the removal of chylomicron remnants and, in the absence of one receptor, the other compensates (2). Studies in LDL receptor and RAP (3) knockout mice have provided strong evidence for this hypothesis (2, 4). A second group of hypotheses revolves around the existence of other receptors. Bihain and Yen (5) have described a protein whose expression is stimulated by the presence of high concentrations of free fatty acids and proposed that it is a remnant receptor. Based on their studies with the compound lactoferrin, van Dijk and colleagues (6) also suggest the existence of another receptor. Utilizing a variety of experimental systems including the apoE knockout mouse, Chang et al. (7) and Borensztajn and colleagues (8–10) have provided evidence for a non-apoE-dependent mechanism of remnant removal that may involve the phospholipase activity of hepatic lipase. The third series of suggestions revolve around the role of other cell surface molecules including glycosaminoglycans (11) and hepatic lipase (11–13) as primary or ancillary ligands, along with the above-mentioned receptors. In particular, it has been suggested that the ability to secrete these molecules as well as apoE results in a process called "secretion capture" (11). Wisse et al. (14) have proposed that the hepatic architecture results in

Abbreviations: LDL, low density lipoprotein; LRP, low density lipoprotein receptor-like protein; RAP, receptor-associated protein; GST, glutathione-S-transferase; HDL, high density lipoprotein; TLC, thin-layer chromatography; CHO, Chinese hamster ovary; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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"sieving" of remnants with their retention in the space of Disse.

Previous work from our laboratory (1, 15, 16) has focused primarily on the first hypothesis. Utilizing the mouse and a variety of receptor antagonists, we have suggested that in the normal animal the LDL receptor plays the largest role in remnant removal and that the LRP plays a smaller role.

The purpose of the present study was to obtain more precise quantitative estimates of the role of each of the lipoprotein receptors and to begin to estimate the roles of ancillary molecules as well as elucidating their mechanism of action. The results provide an assessment of the role of each receptor as well as a role for hepatic lipase.

MATERIALS AND METHODS

Radiochemicals

$Na^{125}I$ (carrier-free) was purchased from Amersham Corp. (Arlington Heights, IL). [9,10-^{3}H(N)]triolein and [9,10-^{3}H(N)]oleic acid were obtained from DuPont NEN Research Products (Boston, MA).

Proteins

A plasmid containing the cDNA for human receptor-associated protein (RAP) fused with glutathione-S-transferase (GST) in *E. coli* was kindly provided by Dr. D. Strickland (17). The protein was purified in our laboratory as described earlier (15). The anti-rat LDL receptor antibodies used have been previously described and characterized (18). Antiserum to rat hepatic lipase fusion protein (19) was prepared in male New Zealand white rabbits by standard procedures utilizing 100 μg of hepatic lipase fusion protein for initial and subsequent injections. Immunoglobulins (IgG) were isolated by use of protein A-agarose (Bio-Rad). Nonimmune IgG was isolated from normal rabbit sera using the same procedure.

Preparation of lipoproteins

Rat mesenteric lymph chylomicrons and chylomicron remnants were prepared in vivo as previously described (20, 21). The chylomicron remnants were labeled by two different procedures. For most of the experiments chylomicron remnants were iodinated by a modification (22) of the iodine monochloride method. The distribution of radioactivity between lipid and protein was monitored on all batches as previously described (23) and fell within the range reported (23). In one experiment, chylomicron remnants were isolated from rats that had been injected with chylomicrons that had been labeled biologically by adding [^{3}H]oleic acid to the duodenal infusion. Human LDL (d 1.019–1.063 g/ml) and high density lipoprotein (HDL) (d 1.063–1.210 g/ml) were isolated from EDTA-containing plasma by sequential ultracentrifugation. Total protein concentration of lipoproteins was determined by the BCA-procedure.

Animals

Female Swiss Webster mice weighing 29–30 g were purchased from Simonsen Laboratories (Gilroy, CA). Male Sprague-Dawley rats from the same source were used either as lymph donors or for functional hepatectomies. The animals had free access to standard chow and tap water.

Measurement of plasma clearance and tissue uptake of chylomicron remnants in mice

Mice were injected in the tail vein with 10 μg protein of ^{125}I-labeled chylomicron remnants in 0.1 ml saline. Blood samples at different time points were collected into heparinized capillary tubes by puncturing the retroorbital plexus (24). Radioactivity in 10 μl of blood was measured in a gamma counter and the amount of lipoprotein remaining in the plasma was calculated as a percentage of the initial blood concentration. The initial plasma concentration for chylomicron remnants was estimated by extrapolating the amount of lipoprotein at time zero from the first two measured values. After the last blood sample was collected, the animals were euthanized and various tissues were removed, rinsed in PBS, and dried in an oven overnight (80°C). The weight and the amount of radioactivity were then measured. Tissue spaces were calculated by the method of Spady, Bilheimer, and Dietschy (25) using the following formula: tissue space (microliters plasma per gram dry tissue weight) = [cpm in tissue/(grams dry weight × cpm/microliters plasma)]. The cpm per microliter of plasma was calculated from the average amount of radioactivity in the 0 and 5 min samples.

Determination of hepatic lipase activity

The lipase activity in the serum or post-heparin plasma was determined by the method of Nilsson-Ehle and Shotz (26) using a gum arabic-stabilized [^{3}H]triolein emulsion in the presence of 1 M NaCl. Rat plasma was diluted with rat serum that was previously incubated at 56°C for 1 h to inactivate any lipase activity present. Lipase activities are given in μmol free fatty acid released/ml sample per h.

In one experiment, ^{3}H-labeled chylomicron remnants were used as a substrate. The triglyceride lipase and phospholipase activities were calculated after thin-layer chromatography (TLC) using three solvent systems: chloroform–methanol–H_2O 65:35:6; ethyl ether–benzene–ethanol–acetic acid 40:50:2:0.2; and ethyl ether–hexane 6:94. The results were expressed both as

the ratios of triglyceride to diglyceride plus free fatty acids and of phospholipids to lysophospholipids or as percentage of control.

For antibody inhibition of mouse and rat plasma hepatic lipase activity, plasma samples were pre-incubated with anti-rat hepatic lipase IgG or non-immune IgG at 4°C for 1 h prior to assay of hepatic lipase activity. The volume of sample to volume of antibody was kept constant at 1:1.

Binding of hepatic lipase to lipoproteins

One hundred seventy-five μg of chylomicron remnants, LDL, or HDL was incubated at 37°C for 1 h with medium harvested from Chinese hamster ovary (CHO) cells that secrete hepatic lipase (19). The mixtures were then exhaustively washed through Amicon Centricones with a molecular weight cutoff of 100 kDa to remove unbound hepatic lipase. Medium incubated in the absence of lipoprotein was included as a control. The relative amount of bound hepatic lipase was determined by immunoblotting. The mixtures derived from the binding experiments were separated by electrophoresis on 10% polyacrylamide gels (PAGE) containing sodium dodecyl sulfate and immunoblotted as described earlier. Briefly, proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was incubated with anti-rat hepatic lipase serum (1:2000) for 1 h at room temperature, washed, incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:1000), and developed as described (19).

A solid phase assay was also used. Microtiter plates (96 wells) were coated with 100 μl of medium from CHO cells secreting hepatic lipase and were dried overnight at 37°C. Parallel wells were coated with medium from non-hepatic lipase-secreting CHO cells to determine nonspecific binding. The wells were washed three times with 200 μl of 0.3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). A 10-fold higher concentration of BSA (3%) in PBS was then added (200 μl) and the plates were incubated at 37°C for 60 min to block nonspecific binding sites, followed by another wash. Antihepatic lipase IgG or nonimmune IgG was added to the hepatic lipase pre-coated wells and incubated at 4°C for 1 h and then ^{125}I-radiolabeled chylomicron remnants in a 25 μl of buffer were added and incubated at 37°C for 3 h. The incubation media were removed and the wells were washed extensively. The amount of radioactivity remaining in each well was measured in a gamma counter.

Statistics

Statistical analysis was done by nonpaired Student's *t* test.

RESULTS

Effect of anti-LDL receptor antibody on the removal of chylomicron remnants from plasma

The anti-LDL receptor antibody used has been extensively characterized (18). Its ability to inhibit lipoprotein binding to the LDL receptor (27) and its lack of cross-reactivity to the LRP (28) and the VLDL receptor (29), as well as its effect in vivo (15, 24), have all been published. Dose–response and time-course studies were performed with the antibody to establish the optimal conditions to maximally block chylomicron remnant removal. At 1 mg anti-LDL receptor antibody per mouse, the maximal inhibitory effect was seen (not shown). Nonimmune IgG at the same concentration had an insignificant inhibitory effect on chylomicron remnant clearance as compared to saline pre-injected mice (not shown). There was no difference in remnant clearance when animals were pretreated 30 min rather than 1 h before injection of chylomicron remnants (not shown). ^{125}I-labeled chylomicron remnants were injected intravenously and plasma clearances were compared in animals pretreated with anti-LDL receptor antibody or nonimmune IgG (**Fig. 1A**). As previously reported (15), chylomicron remnants are cleared very rapidly from the circulation. In 5 min, about 78% of the injected dose disappeared from the plasma. The injected lipoprotein was cleared more slowly in the animals pretreated with the anti-LDL receptor antibody and the difference was statistically significant at each time point. At 5 min, 44% of the injected labeled chylomicron remnants were still in the mouse circulation, an inhibition of about 45% as compared to controls. The large number of animals tested for both control and anti-LDL receptor antibody-treated animals in these experiments resulted in a very small standard error. This facilitated statistical comparison with other treatments.

Effect of anti-LDL receptor antibody on chylomicron remnant uptake by tissues

After the final blood sample, the liver, spleen, adrenal glands, lungs, both kidneys, and a segment of the small intestine were removed and associated radioactivity was determined. The dry tissue weight was measured to reduce effects of moisture. The liver and spleen accumulated most of the chylomicron remnants as compared to the other tissues (Fig. 1B). The anti-LDL receptor antibody reduced the liver uptake by about 40% at 5 min, demonstrating that remnant uptake in this organ is mediated to a significant degree by the LDL receptor. Uptake by the other organs was not significantly affected by the presence of the anti-LDL-receptor antibody.

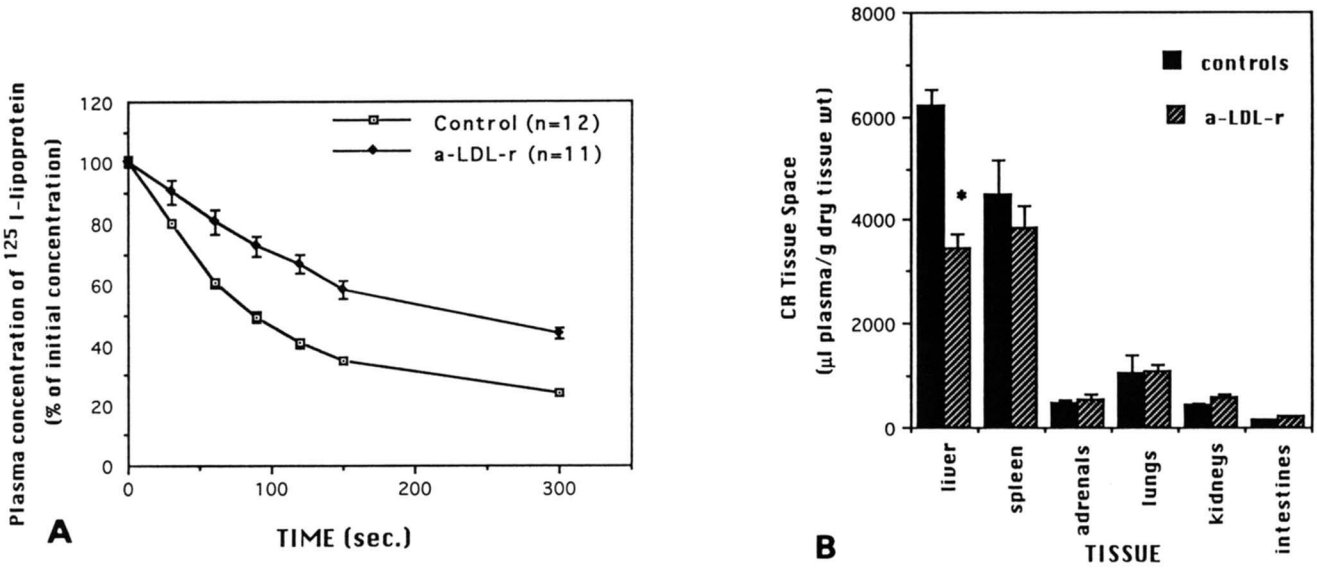


Fig. 1. A: Effect of anti-LDL receptor antibody on the disappearance of ^{125}I-labeled chylomicron remnants from plasma. Mice were injected intravenously with 0.1 ml rabbit anti-LDL receptor IgG (0.5 mg) or saline. After 1 h, they were injected intravenously with 10 µg of ^{125}I-labeled chylomicron remnants (0.01 mg/100 µl) or saline (100 µl) via the lateral tail vein and blood samples (10 µl) were taken for determination of radioactivity. Results are expressed as the amount of radiolabel remaining in the circulation as a percent of the initial plasma concentration calculated as described in Methods. Each point represents the mean ± SE; (▣) ^{125}I-labeled chylomicron remnants after preinjection of non-immune rabbit IgG (n = 12); (◆) ^{125}I-labeled chylomicron remnants after preinjection of rabbit anti-LDL receptor IgG (n = 11). All points were different at $P < 0.01$ or less. B: Effect of anti-LDL receptor antibody on tissue uptake of ^{125}I-labeled chylomicron remnants. At the conclusion of the experiment in Fig. 1A, the animals were killed. Tissues were removed, rinsed in saline, blotted dry, placed in an oven (80°C) overnight, and then weighed. The radioactivity of the tissues was then measured. Tissue spaces were calculated as µl plasma/g dry tissue wt as described in Methods. Results are expressed as mean ± SE. Number of animals is the same as in Fig. 1A. (■) ^{125}I-labeled chylomicron remnants alone; (▨) ^{125}I-labeled chylomicron remnants + anti-LDL receptor antibody; *, $P < 0.005$.

Effect of GST-RAP on the removal of chylomicron remnants from plasma

GST-RAP was used at a high concentration as an LRP and LDL receptor blocking protein. It binds to all of the ligand binding sites of LRP and it is rapidly cleared from the circulation (30). The appropriate protocol was established by dose–response and time-course studies similar to those with the anti-LDL receptor antibody (not shown). GST-RAP at 1 mg per mouse, injected 1 min before the injection of labeled chylomicron remnants, caused a profound inhibitory effect on the plasma removal of chylomicron remnants, caused a profound inhibitory effect on the plasma removal of chylomicron remnants at all time points (**Fig. 2A**). The blockage was significantly greater than by the anti-LDL receptor antibody at each point ($P < 0.02$). As GST-RAP very rapidly disappears from the circulation, the values shown may be minimal estimates of the amount of inhibition.

Effect of GST-RAP on chylomicron remnant uptake by tissues

GST-RAP blocked 75% of chylomicron remnant uptake by the liver (Fig. 2B). This was also significantly greater ($P < 0.01$) than the inhibition by the anti-LDL receptor antibody alone, suggesting either more complete blockade of the LDL receptor, or more likely, blockade of both the LDL receptor and the LRP. Interestingly, RAP substantially decreased uptake of chylomicron remnants by the spleen. This effect is not seen with the LDL receptor antibody and suggests the LRP may play an important role in remnant removal by cells of the reticuloendothelial system.

Combined effect of anti-LDL receptor antibody and GST-RAP on the removal of chylomicron remnants from plasma

When both inhibitors, RAP and anti-LDL receptor antibody, were injected together, the effect on plasma removal was significantly greater than with either inhibitor alone (**Fig. 3A**). Plotted logarithmically (Fig. 3B) the initial removal in the control group had a $T_{1/2}$ of 63 s. Anti-LDL receptor antibody increased the half-life of remnants to 115 s; GST-RAP increased it to 230 s; and in the presence of both the half-life was increased to 290 s. These data support the conclusion that the great majority of the initial disappearance of remnants from the circulation is receptor mediated, at least in the normal state in the mouse.

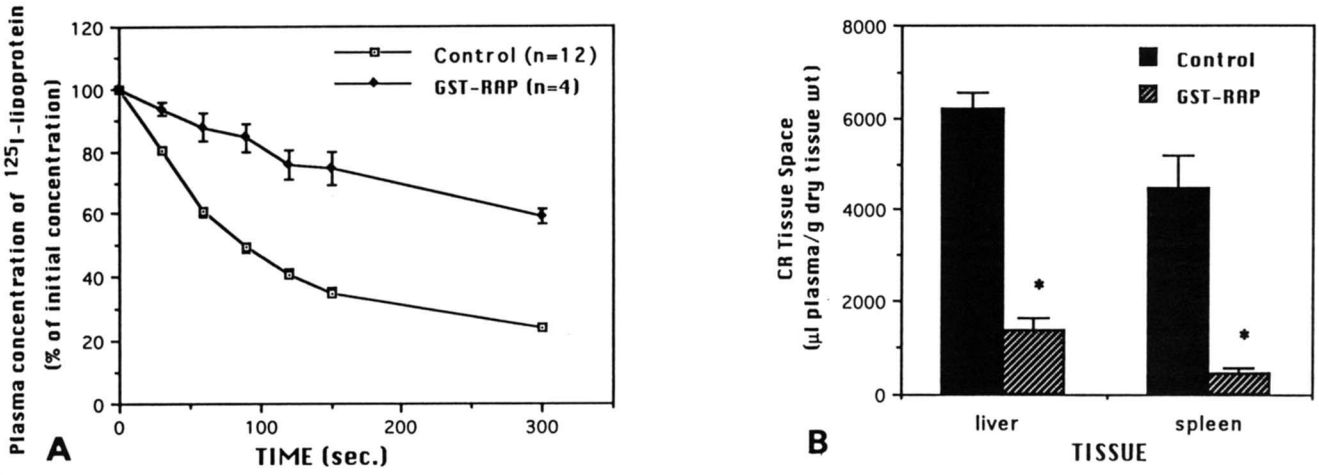


Fig. 2. A: Effect of the receptor-associated protein on the disappearance of ^{125}I-labeled chylomicron remnants from plasma. Mice were injected intravenously with 1 mg recombinant GST-RAP or saline. After 1 min, they were injected intravenously with 10 µg of ^{125}I-labeled chylomicron remnants (10 µg/100 µl) or saline (100 µl) via the lateral tail vein and blood samples (10 µl) were taken for determination of radioactivity. Results are expressed as the amount of radiolabel remaining in the circulation as a percent of the initial plasma concentration, which was calculated as described in Methods. Each point represents the mean ± SE; (⊡) ^{125}I-labeled chylomicron remnants after preinjection of the receptor associated protein (n = 3); (◆) ^{125}I-labeled chylomicron remnants after preinjection of saline (n = 12). All points were different at $P < 0.01$ or less. B: Effect of the receptor associated protein on hepatic uptake or ^{125}I-labeled chylomicron remnants. At the conclusion of the experiment in Fig. 2A the animals were killed. Tissues were treated as described in the legend of Fig. 1B. Hepatic and splenic uptake are shown. Results are expressed as mean ± SE. Number of animals is the same as in Fig. 2A; (■) ^{125}I-labeled chylomicron remnants alone; (▨) ^{125}I-labeled chylomicron remnants + the receptor associated protein; *, $P < 0.01$.

Combined effects of anti-LDL receptor antibody and GST-RAP on chylomicron remnant uptake by the liver

Similarly, most (80%) of the liver uptake of chylomicron remnants was blocked by the combined treatment and was greater than with either alone (Fig. 3C). This also suggests that most of the rapid uptake by liver requires one or both receptors.

Albumin space

The volume of plasma trapped in various tissues was estimated using ^{125}I-labeled albumin as described previously (31) (**Table 1**). The size of the trapped plasma space in the liver accounts for about 7.5% of remnant removal by this organ if one makes the assumption that there is neither preferential exclusion nor concentration of the large remnant particles in this space.

Effect of anti-hepatic lipase antibody on the removal of chylomicron remnants from plasma

Although the above results suggest that most remnant removal in the mouse requires one or both of the LDL receptor family lipoprotein receptors, they do not entirely explain the hepatic specificity of the process, nor do they exclude a role for other molecules as facilitators of the process. Data from several laboratories (11–13) have suggested that hepatic lipase may have a role in lipoprotein uptake; accordingly, we undertook to study this in vivo. An antibody to hepatic lipase was prepared against recombinant rat protein and has previously been characterized (19). The amount of anti-hepatic lipase antibody used in the study was determined by dose–response studies (not shown). The maximal effect was seen with 1.5 mg per mouse injected 1 h before the lipoprotein and injection of twice this amount did not further delay remnant removal or hepatic uptake.

Compared with pretreatment with nonimmune IgG, injection of anti-hepatic lipase antibody inhibited the plasma disappearance of chylomicron remnants injected 1 h later (**Fig. 4A**). The degree of inhibition was modest but statistically significant at all time points ($P < 0.05$); at 5 min, the plasma concentration of labeled remnants was 6% greater in the anti-hepatic lipase antibody group. In comparison to the effect of anti-LDL-receptor antibody (Fig. 4B) or RAP (not shown), the effect of anti-HL was of a significantly lower magnitude ($P < 0.05$ or less). The half-life of chylomicron remnants increased from 63 s to 82 s after anti-hepatic lipase antibody but was still much faster than in the anti-LDL receptor-pretreated mice (115 s). When the two antibodies were injected together, no additive effect was seen on either removal (Fig. 4C) or hepatic uptake (not shown). This is compatible with the hypothesis that the hepatic lipase effect requires the LDL receptor pathway. Similarly, there was no additive effect of anti-hepatic lipase antibody and RAP (not shown).

Effect of anti-hepatic lipase on chylomicron remnant uptake by tissues

Five minutes after the injection of chylomicron remnants, the hepatic uptake of chylomicron remnants (Fig.

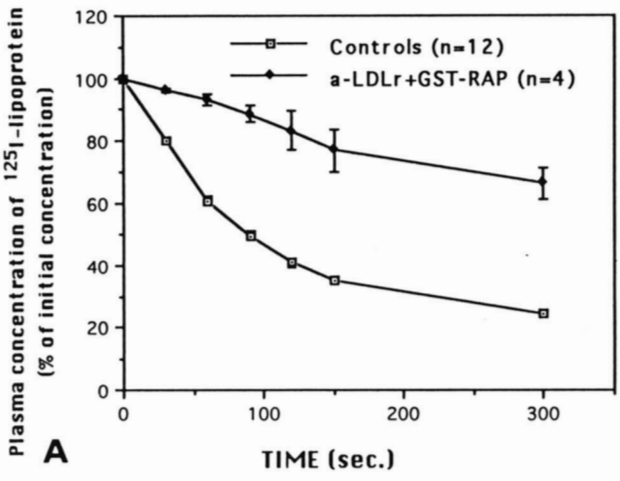
4D) was reduced by 29% in the anti-hepatic lipase-treated mice and the difference was statistically significant. As with plasma removal, there was not an additive effect of anti-hepatic lipase to that of anti-LDL receptor antibody or RAP (not shown). Interestingly, with anti-hepatic lipase antibody treatment, remnant uptake by the adrenal gland increased by 47% as compared to control at 5 min ($P < 0.05$) and the difference was statistically significant (Fig. 4D).

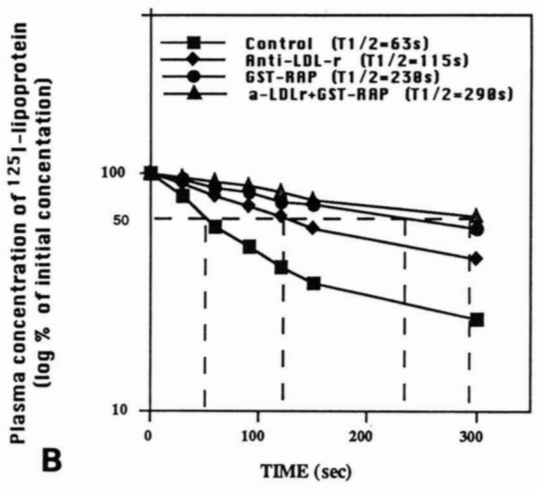
The greater effect on hepatic uptake, as compared to that on plasma removal, along with the effect on the adrenal gland suggests that the blockade of hepatic lipase may allow the uptake of particles by other tissues that have a high concentration of LDL receptors or the LRP.

Effect of anti-hepatic lipase antibody on hepatic lipase activity in rat and mouse serum

To further explore the mechanism of hepatic lipase involvement in remnant removal, the lipolytic activity of hepatic lipase was measured in rat and mouse plasma in the presence or absence of anti-hepatic lipase antibodies (**Table 2A**). The activity of hepatic lipase in mouse post-heparin plasma assayed, using a triolein emulsion, was much lower than in rat post-heparin plasma. Addition of anti-hepatic lipase antibody to rat post-heparin plasma resulted in a profound inhibition of lipase activity. In contrast, the activity in mouse plasma was not inhibited by the anti-hepatic lipase antibody. To exclude an artifact due to the different plasma enzyme concentrations, rat plasma was diluted to the same initial hepatic lipase activity as mouse plasma. The antibody still profoundly inhibited the rat enzyme.

To learn whether the lack of inhibition of mouse hepatic lipase by the anti-rat hepatic lipase antibody extended to physiologic substrates, endogenously labeled chylomicron remnants were utilized in the assay of hepatic lipase activity (Table 2B). Both the neutral lipolytic and the phospholipase activities were inhibited by the antibody with rat plasma as a source of lipase, while the mouse lipase hydrolyzed triglycerides to the same degree despite the presence of the antibody. The phospholipase activity of mouse plasma was slight and not affected by the anti-hepatic lipase antibody. The conclusion that an inhibition of hepatic lipase lipolytic activity is not needed to reduce remnant uptake in the





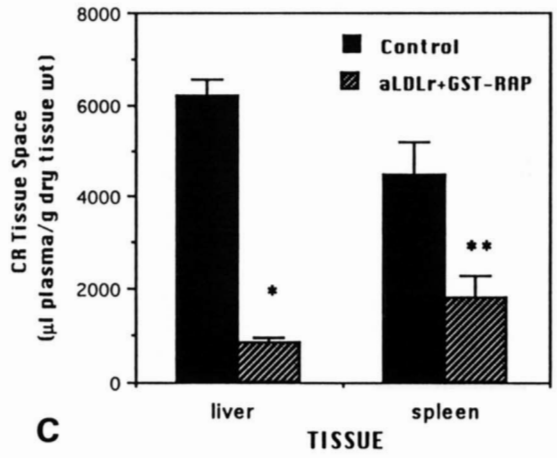


Fig. 3. A: Effect of anti-LDL receptor antibody and the receptor associated protein together on the disappearance of ^{125}I-labeled chylomicron remnants from plasma. An experiment similar to that described in Fig. 2 was conducted except that the animals were pretreated with 0.1 ml rabbit anti-LDL receptor IgG (0.5 mg) or saline 1 h before the injection of RAP. (▫) ^{125}I-labeled chylomicron remnants alone (n = 12); and (◆) ^{125}I-labeled chylomicron remnants + combined anti-LDL receptor IgG and RAP (n = 4). All points were different at $P < 0.01$ or less. B: Logarithmic transformation of the data in Figs. 1A, 2A, and 3A. C: Effect of anti-LDL receptor antibody and the receptor-associated protein together on hepatic uptake of ^{125}I-labeled chylomicron remnants. At the conclusion of the experiment in Fig. 3A the animals were killed. Tissues were treated as described in Fig. 1B. Hepatic and splenic uptake are shown; (■) ^{125}I-labeled chylomicron remnants alone; (▨) ^{125}I-labeled chylomicron remnants + anti-LDL receptor antibody + RAP; $P < 0.01$.

mouse suggests that the effect of hepatic lipase on remnant uptake is mediated by a property other than lipolysis.

Binding of hepatic lipase to lipoproteins

The above result suggests that a role of hepatic lipase may be to modulate or facilitate binding of the chylomicron remnant to the cell surface. To explore this, the ability of hepatic lipase to bind to lipoproteins was studied. Chylomicron remnants, LDL, and HDL were added to media harvested from transfected CHO cells that secrete hepatic lipase. The media were then filtered through Centricone filters and the retained lipoproteins were washed to remove non-bound hepatic lipase and processed for PAGE. The same amount of protein was added to each lane. The presence of hepatic lipase was detected by Western blotting of the lipoproteins studied. All of the lipoproteins bound hepatic lipase. This was greatest for chylomicron remnants (**Fig. 5**). In another assay, the addition of the anti-hepatic lipase antibody did not affect the binding (not shown).

To confirm the results of the immunoblot studies, additional binding studies were performed using a solid phase assay. Plates were precoated with media from hepatic lipase secreting or non-lipase secreting CHO cells. This was followed by the addition of 0.25 μg/well of ^{125}I-labeled chylomicron remnants. The binding of remnants was 45% greater in the wells adsorbed with hepatic lipase containing media (**Table 3**); the increase in binding was found to be dose-dependent with the amount of hepatic lipase (not shown). This is not reduced by the presence of anti-hepatic lipase antibody (Table 3B).

DISCUSSION

The present studies extend our previous work and provide a basis for estimating the contribution and mechanism of action of various molecules in the removal of chylomicron remnants by the liver. Table 4 provides a summary of these estimates. At least 80% of the removal of remnants requires the presence of lipoprotein receptors. This is likely to be a minimum estimate as the studies did not correct for trapped plasma. This is likely to be a significant effect as we did not flush the liver to remove trapped blood, and thus trapped lipoproteins, in the liver. It has been traditional to do this by the use of an albumin space (25). If the albumin space is used in the present studies, the amount of rapid hepatic uptake that is receptor-mediated is estimated at 87.5%. The applicability of the albumin space to the amount of remnants present in the plasma in the liver is not established. Albumin is a small molecule and may penetrate the space of Disse better than large lipoproteins; however, Wisse et al. (14) have suggested that there is a phenomenon whereby remnants may be preferentially "sieved" into this space and would thus be concentrated in it (32). We are currently evaluating the importance, if any, of this phenomenon. Based on the above, however, it is concluded that virtually all of the rapid removal of remnants from the plasma requires the presence of lipoprotein receptors of the LDL receptor family and is a strong confirmation of the two-receptor hypothesis of Ishibashi et al. (2).

It is somewhat more difficult to apportion the contributions of the LDL receptor and the LRP. The anti-LDL receptor antibody used is monospecific and does not affect binding to the LRP (28). On a per microgram of protein basis, its affinity for the LDL receptor in mice is about the same as that of LDL and is only about 5–10% that of apoE-rich lipoproteins (33). It is thus likely that the antibody could not completely inhibit remnant binding to the LDL receptor at the concentration achieved in vivo. The 50% of the lipoprotein receptor-mediated hepatic uptake in the normal mouse that is inhibited by the anti-LDL receptor antibody may therefore be a minimum estimate of the contribution of the LDL receptor. In our studies (15) as well as those of Hussain et al. (34), α-2 macroglobulin inhibited about 10% of remnant removal. Because of uncertainty as to how many discrete sites there are for remnant binding and whether these all have the same affinity and are all inhibited by α-2 macroglobulin, this is also a minimum estimate. A similar result was obtained with the injection of low concentrations of RAP. RAP has now been reported by several groups (33, 35) to bind to both the LRP and the LDL receptor; however, its affinity for the LDL receptor is less than 10% that of LDL on a mass of protein basis, and this is far lower than its affinity for the LRP. Thus the greater effect of RAP at the higher dose and the shorter time point could be due to more complete inhibition of the LRP, to partial inhibition of the LDL receptor, or to both of these phenomena. The

TABLE 1. ^{125}I-labeled albumin tissue spaces in the mouse

Organ	μl Plasma/g Dry Tissue Weight
Liver	462.25 ± 71.47
Spleen	428.00 ± 22.76
Adrenals	414.25 ± 70.50
Lungs	1043.25 ± 99.46
Kidneys	609.25 ± 46.82
Intestines	250.25 ± 45.99

Mice were injected intravenously with ^{125}I-labeled albumin via the tail vein. After 15 min, the animals were killed and tissues were removed, rinsed in saline, blotted dry, placed in an oven (80°C) overnight, and then weighed. The radioactivity of the tissues was then measured. Tissue spaces were calculated as μl plasma/g dry tissue weight as described in Methods (mean ± SE, n = 4).

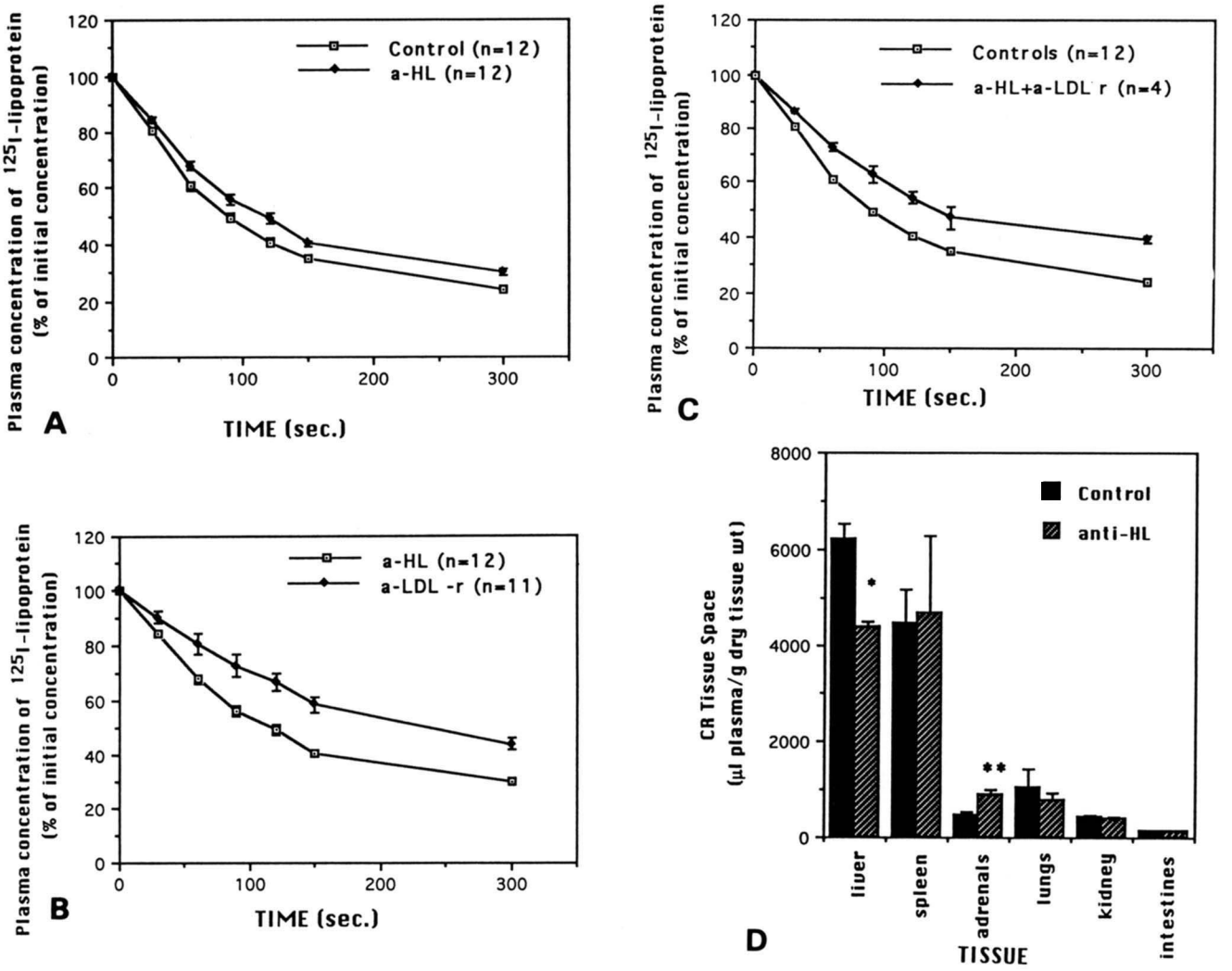


Fig. 4. A: Effect of anti-hepatic lipase antibody on the disappearance of ^{125}I-labeled chylomicron remnants from plasma. The experiment was carried out exactly as described in the legend to Fig. 1A except that a rabbit anti-rat hepatic lipase fusion protein IgG was used. Results are expressed as the mean ± SE; (▣) ^{125}I-labeled chylomicron remnants alone; (◆) ^{125}I-labeled chylomicron remnants + anti-LDL receptor antibody (n = 12 for both groups); $P < 0.05$ or less at each time point. B: Effect of anti-LDL receptor antibody and anti-hepatic lipase antibody together on the disappearance of ^{125}I-labeled chylomicron remnants from plasma. The experiment was carried out exactly as described in the legend to Fig. 1A except that both anti-LDL receptor IgG and anti-rat hepatic lipase fusion protein IgG were injected simultaneously; (▣) ^{125}I-labeled chylomicron remnants alone; (◆) ^{125}I-labeled chylomicron remnants + anti-LDL receptor antibody. Mean IgE is shown (n = 4). The data for anti-LDL receptor antibody alone are not shown because they are superimposable on the upper curve. C: Comparison of the effects of anti-hepatic lipase antibody with that of anti-LDL receptor antibody. The data of Figs. 1A and 5A are compared; $P < 0.01$ or less at each time point. D: Effect of anti-hepatic lipase antibody on tissue uptake of ^{125}I-labeled chylomicron remnants. Tissue uptake of ^{125}I-labeled chylomicron remnants by the animals described in the legend of Fig. 5A was determined as described in the legend to Fig. 1B; (■) ^{125}I-labeled chylomicron remnants alone; (▨) ^{125}I-labeled chylomicron remnants + antihepatic lipase IgG.

increment in inhibition when RAP and the anti-LDL receptor are injected simultaneously could be due to achieving complete inhibition of the LDL receptor, which cannot be accomplished with either reagent alone. If this is the case, it would suggest that the minimum amount taken up by the LDL receptor is 55% and that by the LRP is 20%. Alternatively, most of the inhibition could be due to inhibition of the LRP, making the LRP overall contribution a maximum of 40%.

The RAP molecule has a heparin binding site (36) but had been reported not to bind (37) to cell surface proteoglycans. Recently this was disputed (38). The affinity of this reaction and thus whether it is relevant to our studies is not clear. If some of the RAP effect is due to blocking this binding, it would decrease the amount ascribed to LRP and increase that due to non-receptor-mediated binding.

The precise quantitative roles of each receptor will require further refinement in the methodology and will always be subject to variation due to metabolic factors. However, taken together, these data provide an increased precision of the estimate of the relative roles of

TABLE 2. Effect of anti-rat hepatic lipase antibody on hepatic lipase activity in the rat and mouse

	Hepatic Lipase Activity	
	Non-immune IgG	Anti-hepatic Lipase IgG
	μmol fatty acid/ml/h	
A: Triolein emulsion[a]		
Rat plasma	19.2 ± 2.2 (4)	5.3 ± 0.7 (4)
Rat plasma (diluted 1:4)	5.2 ± 0.9 (3)	2.6 ± 0.2 (3)
Mouse sera	3.8 ± 0.3 (4)	4.4 ± 0.4 (3)

	TG + DG/FFA		PL/Lyso PL	
	Ratio	% Control	Ratio	% Control
B: Radiolabeled chylomicrons[b]				
Additions to chylomicron remnants				
None	33	100	1.20	100
Rat plasma	15	45	0.85	71
Rat plasma + anti-hepatic lipase IgG	22	67	1.22	100
Mouse plasma	23	70	1.30	110
Mouse plasma + anti-hepatic lipase IgG	20	61	1.08	90

[a]Mouse post-heparin plasma or undiluted or diluted rat post-heparin plasma was incubated with a triolein emulsion and the amount of free fatty acid liberated was determined as described in Methods. The incubations were carried out in the presence of either non-immune rabbit IgG or IgG from rabbits immunized with a rat hepatic lipase fusion protein; mean ± SE.

[b]Rats fitted with cannulated lymph ducts were given [^{3}H]oleic acid and chylomicrons were isolated from their lymph. Chylomicron remnants were prepared from the endogenously labeled chylomicrons and incubated with rat or mouse post-heparin plasma in the presence or absence of non-immune rabbit IgG or anti-rat hepatic lipase IgG. Triglycerides (TG), diglycerides (DG), free fatty acids (FFA), phospholipids (PL), and lysophospholipids (Lyso PL) were isolated and quantified as described in Methods. The actual ratio is on the left and the % of the ratio to that in native chylomicrons is on the right of each pair.

the two receptors and establish receptor binding as a prerequisite for remnant removal.

In a recent publication describing the RAP knock-out mouse, Willnow et al. (3) noted no accumulation of remnants despite a marked reduction in LRP levels. Herz et al. (39) did find a profound decrease in remnant internalization in the LDL receptor knock-out mouse. Their observations are consistent with the postulate that in the normal animal the LDL receptor plays a large role in the rapid removal of remnants. When the LDL receptor is absent, it is clear that some abnormality of remnant removal occurs (40, 41). Interestingly, when studied at later time points, removal but not internalization of remnants often appears to be normal (39). This contrasts with the anti-LDL receptor antibody data of this and our previous publications. It suggests the possibility that in the chronic absence of LDL receptors, compensatory increases in other factors may develop or that secondary mechanisms including the LRP, hepatic lipase, and glycosaminoglycans will remove remnants but do so more slowly. There are several additional implications of this. When comparing studies, careful attention must be paid to the time points chosen to measure clearance from plasma and hepatic uptake. At later time points, the role of the higher affinity processes may not be fully appreciated because eventually the lower affinity process will compensate for the loss of the higher affinity process. Thus, even in the LDL receptor knockout with blocked or reduced LRP models of Willnow and colleagues (3, 4), remnants are eventually removed.

Clinically, this consideration may be important as well. Recent evidence has established that delayed removal of diet-derived lipoprotein is a risk factor for atherosclerosis (42, 43). Thus shifting from LDL-mediated to LRP-mediated to non-receptor-mediated processes for remnant removal may not result in morbid accumulation of remnants in the circulation under most circumstances but in an increased residence.

An interesting difference between anti-LDL receptor antibody and RAP was noted for splenic uptake, which was inhibited by RAP and not by anti-LDL receptor antibody. Hussein et al. (44) have previously suggested that uptake in reticuloendothelial-rich tissues can be quantitatively significant in some species. These data suggest that such uptake is mediated by the LRP, although an effect of RAP on the scavenger receptor was not excluded. If rapid uptake by tissue macrophages is

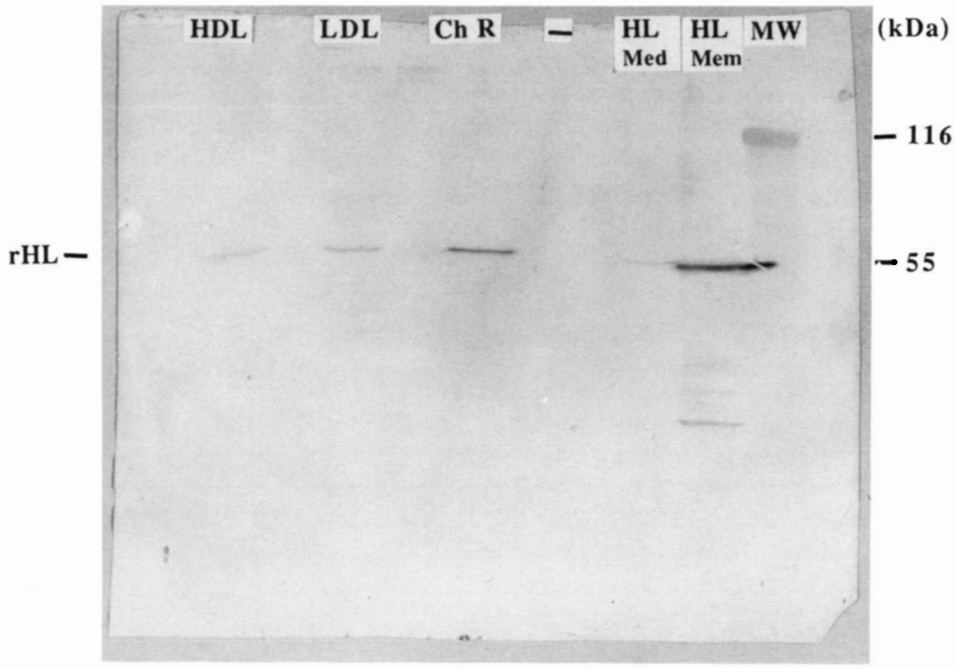


Fig. 5. Binding of hepatic lipase to lipoprotein. Chylomicron remnants, LDL, and HDL were prepared as described in Methods. One hundred seventy-five mg of lipoprotein protein was incubated at 37°C for 1 h with 0.5 ml medium from CHO cells transfected to secrete hepatic lipase. The mixtures were then placed in centricone filters with a mol wt cut-off of 100 kDa and washed exhaustively. The residual lipoprotein was then subjected to PAGE, transferred to nitrocellulose, and this was probed with rabbit anti-hepatic lipase antibody and developed with HRP-conjugated goat anti-rabbit IgG. HDL, high density lipoprotein; LDL, low density lipoprotein; Ch R, chylomicron remnants;—,no lipoprotein added to medium; HL Med, medium from transfected CHO cells before addition of lipoproteins; HL Mem, membranes from transfected CHO cells; MW, molecular weight marked as indicated; rHL, the molecular weight of rat hepatic lipase. A representative experiment is shown.

mediated by the LRP, this would provide a mechanism whereby remnants could directly contribute to foam cell formation. Because of its size, the quantitative information of splenic uptake is small.

If virtually all of remnant removal requires lipoprotein receptors, even for initial clearance, what is the role, if any, of other molecules? Hepatic lipase was chosen as a model to address this for several reasons. It has been found that in familial hepatic lipase deficiency, particles of a remnant-like nature accumulate (45), and injection of anti-hepatic lipase antibody into rats caused accumulation of such particles (12). In addition, tissue culture studies have suggested that the molecule can accelerate the uptake of several lipoprotein species. Ji et al. (11) showed this for β very low density lipoprotein in a hepatoma line, and we (16) found this for LDL but not chylomicron remnants in a transfected CHO cell line. The lack of an effect with remnants may have been due to the non-hepatic cell type used in the latter experiments. Thus, it was not surprising that in the mouse, as has been reported in the rat (13), remnant removal was delayed by pretreatment with an anti-hepatic lipase antibody.

It is our hypothesis that this occurs because hepatic lipase provides additional low affinity binding sites for remnants. These, in conjunction with lipoprotein receptors, increase the K_d of the particle for the cell surface. In this postulate, the capacity for initial remnant removal is determined by the number of lipoprotein receptors, while the affinity is determined by the conjoint action of numerous cell surface receptors. Several lines of evidence support this formulation. First, the effects of the anti-hepatic lipase antibody and anti-LDL receptor antibody or RAP were not additive. Even though the effect of anti-hepatic lipase antibody alone was small, the number of animals studied should have allowed detection of even a small additive effect as it did in the RAP and LDL receptor antibody combination studies. Second, the effect on clearance from the blood was less than that on uptake by the liver. This, complemented by the increase in uptake by the adrenal glands, the other organ studied that expresses a high level of LDL receptors, suggests that the presence of hepatic lipase gives hepatic LDL receptors an advantage compared to those in the periphery. Third, in our cell culture studies, where the presence of hepatic lipase increased LDL uptake, it

TABLE 3. Solid phase assay for binding of chylomicron remnants to hepatic lipase

	Chylomicron Remnant Binding
	ng protein/well
A:[a]	
Control CHO media	6.8 ± 1.35 (n = 6)
Hepatic lipase-secreting CHO media	11.7 ± 3.35 (n = 9)
B:[b]	
Control CHO media	8.4 ± 0.9 (n = 6)
Hepatic lipase-secreting CHO media + non-immune IgG	12.3 ± 1.8 (n = 6)
Hepatic lipase-secreting CHO media + anti-hepatic lipase IgG	12.0 ± 1.1 (n = 6)

[a]Microtiter plates were coated with 100 μl of medium from either CHO cells secreting hepatic lipase or from non-hepatic lipase secreting cells. ^{125}I-radiolabeled chylomicron remnants were added. After the incubation, the amount of radioactivity was measured as described in Methods.

[b]The same experiment as in A except that the non-immune rabbit IgG or rabbit anti-hepatic lipase IgG was added to the wells before the remnants. Two separate experiments are shown; n, number of replicates.

did so by increasing the affinity for the LDL receptor rather than the number of binding sites.

Consistent with this hypothesis was the somewhat unexpected finding that anti-hepatic lipase antibody did not affect the lipolytic activity of the mouse enzyme for either triglyceride or phospholipid. This does not support the hypothesis that the phospholipase activity is necessary for remnant uptake (8–10). Recently, Diard et al. (46), using heat-inactivated hepatic lipase, have demonstrated a similar effect on the uptake of artificial chylomicron remnant-like particles. Binding of the lipase to chylomicron remnants was readily demonstrated by two different experimental approaches and this is consistent with a mechanism of action that involves alteration of binding characteristics. The anti-hepatic lipase antibody did not alter this binding. It is thus our working hypothesis that the antibody prevents association with the cell surface.

The modest effect of hepatic lipase seen in this study may understate its role in other species, in as much as the mouse is a relatively poor model for studies of hepatic lipase. The levels of hepatic lipase are low in this species as compared to the rat and human, and mouse lipase has a lower affinity for heparin than hepatic lipase from other species. This presumably accounts for a smaller proportion being bound to the liver and probably explains the smaller effect of the anti-hepatic lipase antibody on clearance observed in the mouse as compared to the rat.

A mechanism for enhancement of the affinity of a ligand for the cell surface induced by the presence of ancillary binding sites has precedent in other systems where multifooted binding occurs. Perhaps the best studied of these is binding of antibodies to ligands. The intact antibody has an affinity that is the product of the affinities of the Fab fragments for the same ligand; thus, multifooted binding enhances affinity in a multiplicative rather than an additive manner. Similarly, it has been proposed that the affinity of apoE-containing lipoproteins for the LDL receptor is an order of magnitude higher than for apoB-containing lipoproteins because the multiple copies of apoE on the lipoprotein each bind to multiple LDL receptors (47). The present proposal would extend that concept and suggests that binding to other cell surface molecules, such as hepatic lipase, would allow additional multifooted binding and thus significantly increase the affinity of the lipoprotein for cells that express both low and high affinity sites as compared to those that express only high affinity sites. This model is compatible with the premise that the low affinity site alone is not adequate to mediate binding, at least in vivo, and thus explains the lack of an additive effect of the anti-hepatic lipase and anti-LDL receptor antibodies.

TABLE 4. Estimate of the relative contribution of each receptor to the uptake of chylomicron remnants

	LDL-R		LRP		Non-receptor
	Min	Max	Min	Max	Max
No correction for trapping of plasma	45	70	12.5	37.5	20
Correction for trapping of plasma using albumin	50	76	17	23	13

Estimate of the relative contribution of each receptor to the uptake of chylomicron remnants by the normal mouse liver. These data are derived from the results of the experiments shown in Figs. 1B, 2B, 3C, and Table 1.

In summary; the present studies suggest a model where virtually all of the rapid removal of remnant lipoproteins requires the presence of either the LDL receptor or the LRP. In the normal mouse, the largest portion of clearance relies on the LDL receptor, consistent with its higher affinity for lipoproteins. However, it is evident that if LDL receptors are absent or reduced, as in familial hypercholesterolemia or the LDL receptor knock-out mouse, the LRP should be able to compensate with a modest delay in remnant removal. Hepatic lipase appears to facilitate remnant removal by the liver by providing an additional binding site. The affinity of this site does not appear to be adequate to mediate removal alone, but acts by enhancing the affinity of the particle for the primary receptor on the hepatocyte by allowing multifooted binding. Other cell surface molecules could play a similar role and together these would create the unusually high affinity remnants have for the liver.

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