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# Recognition of chylomicron remnants and β-migrating very-low-density lipoproteins by the remnant receptor of parenchymal liver cells is distinct from the liver $\alpha_2$-macroglobulin-recognition site

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The uptake *in vivo* of chylomicrons and β-migrating very-low-density lipoprotein (β-VLDL) by rat liver, which is primarily carried out by parenchymal cells, is inhibited, 5 min after injection, to respectively 35 and 8 % of the control values after preinjection of lactoferrin. The decrease in the uptake of lipoproteins by the liver caused by lactoferrin is a specific inhibition of uptake by parenchymal cells. Competition studies *in vitro* demonstrate that chylomicron remnants and β-VLDL compete for the same recognition site on parenchymal cells. Data obtained *in vivo* together with the competition studies performed *in vitro* indicate that chylomicron remnants and β-VLDL interact specifically with the same remnant receptor. Hepatic uptake of $^{125}$I-labelled-$\alpha_2$-macroglobulin *in vivo*, mediated equally by parenchymal and endothelial cells, is not decreased by preinjection of lactoferrin and no effect on the parenchymal-cell-mediated uptake is found. *In vitro*, $\alpha_2$-macroglobulin and chylomicron remnants or β-VLDL show no cross-competition. Culturing of parenchymal cells for 24–48 h leads to a decrease in the cell association of $\alpha_2$-macroglobulin to 26 % of the initial value, while the cell association of β-VLDL with the remnant receptor is not influenced. It is concluded that β-VLDL and chylomicron remnants are recognized by a specific remnant receptor on parenchymal liver cells, while uptake of $\alpha_2$-macroglobulin by liver is carried out by a specific receptor system (presumably involving the LDL-receptor-related protein) which shows properties that are distinct from those of the remnant receptor.

## INTRODUCTION

Chylomicrons are relatively large triacylglycerol-rich lipoproteins which transport exogenous lipids. Interaction of chylomicrons with endothelial cell-bound lipoprotein lipase and high-density lipoprotein (HDL) results in the formation of chylomicron remnants. This lipoprotein is relatively depleted of triacylglycerols and phospholipids and enriched with cholesterol, cholesterol esters and protein (Mjøs *et al.*, 1975; Redgrave & Small, 1979). Remnants are rapidly cleared by the liver and uptake is predominantly carried out by parenchymal cells (Groot *et al.*, 1981; Blomhoff *et al.*, 1982, 1985; Jones *et al.*, 1984). As shown by studies in patients with type II hyperlipoproteinaemia (Hoeg *et al.*, 1985) and in WHHL rabbits (Kita *et al.*, 1982), which both possess genetically defective low-density lipoprotein (LDL) receptors, uptake of chylomicron remnants proceeds normally, leading to the conclusion that a receptor system distinct from the LDL receptor, tentatively called the remnant receptor, must be resposible for the avid uptake by liver.

The recognition site for chylomicron remnants differs from the classic LDL receptor (Brown & Goldstein, 1986) in being resistant to regulation (Arbeeny & Rifici, 1984; Krempler *et al.*, 1987), independent of $Ca^{2+}$ and recognizing only apolipoprotein E (apoE) and not apoB (Cooper *et al.*, 1982; Krempler *et al.*, 1987). β-Migrating very-low-density lipoproteins (β-VLDL) are cholesterol-enriched lipoproteins which accumulate in the plasma of patients with type III hyperlipoproteinaemia and cholesterol-fed animals (Frederikson *et al.*, 1969; Shore *et al.*, 1974; Mahley & Holcombe, 1977). Like chylomicron remnants, β-VLDL is rapidly cleared by liver parenchymal cells (Harkes *et al.*, 1989). Studies with β-VLDL *in vitro* indicate that uptake of this lipoprotein by macrophages of human and mouse origin is mediated by an (unusual) LDL receptor (Koo *et al.*, 1986; Ellsworth *et al.*, 1987). Rat liver parenchymal cells hardly express active LDL receptors at all (Harkes & van Berkel, 1984*a*,*b*) and, because the specific β-VLDL interaction with parenchymal cells is not blocked by an excess of LDL, we concluded that, in rats, β-VLDL is cleared from the blood circulation by the remnant receptor (Harkes *et al.*, 1989).

Lactoferrin, an $M_r$-76500 glycoprotein, possesses a cluster of four arginine residues at the *N*-terminus, just like the arginine-rich domain of apoE. Recently, lactoferrin was shown to inhibit the uptake of $^{125}$I-labelled chylomicron remnants by whole liver by 50 % (Huettinger *et al.*, 1988). In the present study we used lactoferrin as a competitor in order to analyse the relationship between uptake sites for chylomicrons and β-VLDL in rat liver *in vivo*. The molecular nature of the remnant receptor is under intensive investigation (Herz *et al.*, 1988; Kowal *et al.*, 1989, 1990; Jaeckle *et al.*, 1989; Lund *et al.*, 1989; Strickland *et al.*, 1990; Beisiegel *et al.*, 1989). Herz *et al.* (1988) identified a 500 kDa protein, the structure of which is closely related to that of the LDL receptor [LDL-receptor-related protein (LRP)] and suggested that this protein may function as a remnant receptor. Subsequently Kowal *et al.* (1989, 1990), Lund *et al.* (1989) and Beisiegel *et al.* (1989) provided additional evidence that LRP can bind apoE, although, *in vitro*, enrichment of β-VLDL with apoE is necessary for recognition.

Recently, it was shown that LRP and the α-macroglobulin

Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein; β-VLDL, β-migrating very-low-density lipoprotein; apoE, apolipoprotein E; LRP, LDL-receptor-related protein; PBS, phosphate-buffered saline (see the Materials and Methods section); DMEM, Dulbecco's modified Eagle medium; 4-APP, 4-aminopyrazolo[3,4-*d*]pyrimidine.

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receptor (Strickland *et al.*, 1990) showed protein sequence homologies and it was concluded that LRP and the $\alpha_2$-macroglobulin receptor are the same molecule. In the present study we looked simultaneously at the chylomicron/$\beta$-VLDL and $\alpha_2$-macroglobulin interactions with the liver in order to analyse directly to what extent the lipoprotein-uptake mechanism can be performed by the LRP/$\alpha_2$-macroglobulin system.

## MATERIALS AND METHODS

### Chemicals

Collagenase types A and D (EC 3.4.24.3), Pronase, cholesterol esterase (EC 3.1.1.13) and foetal calf serum were from Boehringer-Mannheim. Benzamidine, phenylmethanesulphonyl fluoride and polyethylene were from Merck. BSA (fraction V), 17$\alpha$-ethinyloestradiol, heparin (sodium salt, grade I) and 3,3'-diaminobenzidine were from Sigma. Human lactoferrin was from Serva. Metrizamide was obtained from Nycomed A/S, Oslo, Norway. Culture media were from Gibco. 4-Aminopyrazolo[3,4-*d*]pyrimidine was from Janssen, Beerse, Belgium. $^{125}$I (carrier-free) in NaOH and $^3$H-labelled vitamin A were from Amersham. DEAE-Sepharose fast flow, Sephacryl S-300 Superfine, metal-chelating Sepharose fast flow and Mono Q were from Pharmacia. All other chemicals were of analytical grade.

### Animals

Male Wistar rats of mass 225–300 g, fed *ad libitum* with regular chow, were used in this study. For determination of liver uptake and serum decay, rats were starved for 16 h. For isolation of $\beta$-VLDL, six to eight rats of mass 200–220 g were maintained for 16 days on a cholesterol-rich chow (Hope Farms, Woerden, The Netherlands) which included 2% (w/w) cholesterol, 5% (w/w) olive oil and 0.5% (w/w) cholic acid. When indicated, 17$\alpha$-ethinyloestradiol in propylene glycol at a dose of 5 mg/kg body weight (Chao *et al.*, 1979) was injected subcutaneously every 24 h for 3 days. Rats were used 72 h after the first treatment.

### Lipoproteins and $\alpha_2$-macroglobulin

Chylomicrons were isolated from rat lymph, obtained via cannulation of the thoracic duct. Briefly, rats were anaesthetized with diethyl ether and the main intestinal lymph duct was cannulated with silastic tubing (external diameter 0.06 cm, internal diameter 0.03 cm). The duodenum was cannulated and rats were placed in restraining cages. A lipid emulsion (Intralipid; Vitrum, Stockholm, Sweden; supplemented with 1% cholesterol) was infused through the intestinal tubing at a rate of 1.2–1.5 ml/h. At 4 h after surgery, lymph was collected on ice in a plastic tube containing 0.1 ml of 0.1 M-EDTA. Chylomicrons were isolated from rat lymph by ultracentrifugation for 1 h at 40000 rev./min in a Beckman SW40 rotor at 4 °C, washed three times with 8 mM-phosphate-buffered saline (PBS) containing 1 mM-EDTA, pH 7.4.

For isolation of $^3$H-labelled vitamin A-labelled chylomicrons, 2 ml of the lipid emulsion, supplemented with 1% (w/v) cholesterol, was mixed with solvent-free $^3$H-labelled vitamin A (0.3 mCi) (Blomhoff *et al.*, 1985). At 4 h after surgery, the radioactive lipid emulsion was infused into the rats and lymph was isolated as described above.

Remnants were isolated from functionally hepatectomized rats (Redgrave, 1970; Mjøs *et al.*, 1975). To minimize the endogenous VLDL level in recipient animals, the rats (starved for 16 h) were pretreated with 4-APP (solubilized in Tris/HCl buffer, pH 3.5; 30 mg/kg body weight) 5.5 h before surgery, and injected intraperitoneally (Groot *et al.*, 1981). At 2 min before functional hepatectomy, 200 units of heparin/kg body weight was administered to stimulate processing of chylomicrons to remnants. Chylomicron triacylglycerol (10–15 mg; unlabelled, $^{125}$I-, or $^3$H-labelled vitamin A-labelled chylomicrons) was injected into the bloodstream and 30 min later blood was collected by puncture of the abdominal aorta. The radioactivity in the liver was counted and never exceeded 1% of the injected dose. Blood was allowed to clot for 30 min at 25 °C. Serum was prepared by centrifugation for 10 min at 3000 rev./min. $^{125}$I-labelled chylomicron remnants and unlabelled chylomicron remnants were isolated according to Redgrave *et al.* (1975). The upper 1 ml of the KBr gradient ($d \leqslant 1.006$) was isolated by tube slicing and remnants were dialysed against PBS/EDTA, pH 7.4, at 4 °C for 20 h, with repeated changes of buffer.

$\beta$-VLDL was obtained from rats that were starved for 20 h and blood was collected by puncture of the abdominal aorta. The sera were pooled and $\beta$-VLDL was isolated as described (Harkes *et al.*, 1989).

Human LDL ($1.019 < d < 1.063$) was isolated from starved human serum by two repetitive centrifugations according to the procedure of Redgrave *et al.* (1975). LDL was removed by tube slicing and was free of apoE (Harkes *et al.*, 1989).

Human $\alpha_2$-macroglobulin was isolated from fresh human EDTA–plasma essentially according to the method of Sottrup-Jensen *et al.* (1980) with slight modifications introduced by Lomberg-Holm *et al.* (1987) using $Zn^{2+}$ chelate affinity chromatography. Plasma was adjusted to 5 mM-benzamidine and 2 mM-phenylmethanesulphonyl fluoride to inactivate proteolytic activity. Programmable f.p.l.c. and column media for performance at high flow rates were used. $\alpha_2$-Macroglobulin was activated with 0.2 M-methylamine (final concentration) for 2 h and dialysed against PBS/EDTA, pH 7.4, at 4 °C.

### Labelling of lipoproteins

Chylomicrons, $\beta$-VLDL, LDL and $\alpha_2$-macroglobulin were radioiodinated at pH 10 with carrier-free $^{125}$I according to a modification (Van Tol *et al.*, 1978) of the ICl method (McFarlane, 1958). Free $^{125}$I was removed by Sephadex G-25 gel filtration, followed by dialysis against PBS/EDTA, pH 7.4, for 20 h at 4 °C, with repeated changes of buffer. The distribution of radioactivity in labelled chylomicron remnants (determined after processing of chylomicrons into remnants) was $77.3 \pm 7.3$% in protein, $17.9 \pm 4.5$% in lipid and $6.2 \pm 2.3$% unbound. For $\beta$-VLDL, $83.3 \pm 3.3$% was in protein, $12.5 \pm 4.0$% in lipid and $4.8 \pm 1.9$% unbound.

The distribution of the protein-associated radioactivity over the various apolipoproteins was determined by electrophoresis of 5 $\mu$g of $^{125}$I-labelled remnants or 5 $\mu$g of $^{125}$I-labelled $\beta$-VLDL on SDS/5–20% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, and identified protein bands were cut out and counted for radioactivity. For chylomicron remnants, it was found that $16.3 \pm 5.3$% of the protein-associated radioactivity was associated with apoprotein (apo) B, $4.3 \pm 0.5$% with apoA-IV, $5.0 \pm 0.8$% with apoE, $45.8 \pm 5.3$% with apoA-I and $28.7 \pm 4.0$% with apoCs. For $\beta$-VLDL $33.8 \pm 4.5$% was associated with apoB, $1.9 \pm 0.2$% with apoA-IV, $8.7 \pm 3.1$% with apoE, $12.4 \pm 4.9$% with apoA-I and $46.1 \pm 3.1$% with apoCs.

### Serum decay and liver uptake of (lipo)proteins

Rats were anaesthetized by intraperitoneal injection of 15–20 mg of sodium pentobarbital and the abdomens opened. Radiolabelled (lipo)proteins were injected via the inferior vena cava. When indicated, rats received an injection of lactoferrin (70 mg/kg body weight) 1 min before injection of radiolabelled

(lipo)protein. At the indicated times, blood samples of 0.3 ml were taken from the inferior vena cava and allowed to clot for 30 min. The samples were centrifuged for 2 min at 16000 *g* and 100 $\mu$l serum samples were counted. The total amount of radioactivity in the serum was calculated using the equation (Bijsterbosch *et al.*, 1989):

$$\text{Serum volume (ml)} = [0.0219 \times \text{body weight (g)}] + 2.66$$

At the indicated times, liver lobules were excised, weighed and radioactivity was counted. In the case of $^3$H-labelled vitamin A label, liver and tissue samples were burned in a Packard TriCarb 306 sample oxidizer; this always yielded a recovery of $\geqslant 97\%$ label. At the end of the experiment the remainder of the liver was removed, weighed and radioactivity was determined.

The amount of liver tissue tied off at the end of the experiment did not exceed 15% of the total liver weight. Radioactivity was corrected for the radioactivity in plasma assumed to be present in the tissue at the time of sampling (85 $\mu$l/g wet weight) (Caster *et al.*, 1955).

### Isolation of liver cells

Rats were anaesthetized and injected with the radiolabelled (lipo)protein. If indicated, rats received an injection of lactoferrin (70 mg/kg body weight) into the bloodstream 1 min before injection of (lipo)proteins. Rat liver parenchymal, endothelial and Kupffer cells were isolated by differential centrifugation and counterflow elutriation as described in detail elsewhere (van Berkel *et al.*, 1987). The contributions of the different liver cell types to the uptake of (lipo)proteins were determined with the assumption that 92.5% of total liver protein obtained was from parenchymal cells, 3.3% from endothelial cells and 2.5% from Kupffer cells (Blouin *et al.*, 1977; Nagelkerke *et al.*, 1983). Kupffer and endothelial cells were more than 95% pure, as judged from peroxidase staining [0.1% 3,3'-diaminobenzidine in 0.05 M-Tris/HCl, 7% (w/v) sucrose, 0.03% (v/v) $H_2O_2$, pH 7.4; 20 min at 37 °C].

For studies *in vitro*, parenchymal liver cells were isolated by perfusion of the liver with 0.1% collagenase (type D) by the method of Seglen (1976) modified as previously described (Casteleijn *et al.*, 1986). The parenchymal cells obtained (viability $\geqslant 95\%$, and $\geqslant 99\%$ pure) were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 2% (w/v) BSA, pH 7.4.

### Culture of rat hepatocytes

Rat parenchymal cells, isolated according to the procedure of Seglen as described above, were cultured and seeded on 12-well culture plates (Costar) in Williams E medium containing 10% (v/v) foetal calf serum (Kuiper *et al.*, 1989).

### Studies *in vitro* with freshly isolated parenchymal cells

For studies *in vitro*, 2–3 mg of rat liver parenchymal cell protein ($\geqslant 95\%$ viable, as judged by 0.2% Trypan Blue staining) was incubated with 5.0 $\mu$g of radiolabelled lipoproteins/ml or the 3.6 nM-radiolabelled $\alpha_2$-macroglobulin, and indicated amounts of competitor in 0.5 ml of DMEM supplemented with 2% BSA, pH 7.4.

Incubations were carried out in plastic Eppendorf tubes for 10 min at 37 °C with repeated shaking. After incubation, the cells were centrifuged at 50 *g* for 1 min at 4 °C, washed twice with washing buffer [0.9% (w/v) NaCl, 1 mM-EDTA, 0.05 M-Tris/HCl, 5 mM-$CaCl_2$, 0.2% (w/v) BSA, pH 7.4] and twice with washing buffer without BSA. Cells were lysed in 1 ml of 0.1 M-NaOH and subsequently radioactivity and protein content were determined.

### Studies *in vitro* with cultured parenchymal cells

Cultured parenchymal cells were transferred to 4 °C and washed twice with Williams E supplemented with 2% BSA. Cells were incubated for 4 h with 10 $\mu$g of $^{125}$I-labelled $\beta$-VLDL/ml in the presence or absence of 300 $\mu$g of unlabelled LDL/ml, or 3.6 nM-$^{125}$I-labelled $\alpha_2$-macroglobulin, in medium supplemented with 2% BSA. During the incubation the air was saturated with carbogen (95% $O_2$/5% $CO_2$). After incubation the cells were washed three times with washing buffer and three times with washing buffer without BSA. Cells were lysed in 1 ml of 0.1 M-NaOH, and radioactivity and protein content was determined.

### Lipoprotein composition

Lipid composition of lipoproteins was determined using Boehringer enzymic kits for triacylglycerols (no. 701882), phospholipids (no. 691844) and free cholesterol (no. 310328). Esterified cholesterol was determined by adding 20 $\mu$g of cholesterol esterase after determination of free cholesterol. Intralipid (Boehringer) was used as an internal standard. Protein contents were determined according to Lowry *et al.* (1951) with BSA as an internal standard.

For chylomicrons, composition was as follows: $91.3 \pm 0.9\%$ triacylglycerols, $5.6 \pm 1.1\%$ phospholipids, $0.7 \pm 0.2\%$ cholesterol esters, $1.3 \pm 0.3\%$ free cholesterol and $2.1 \pm 0.3\%$ protein. Composition of chylomicron remnants was $68.3 \pm 1.6\%$ triacylglycerols, $8.6 \pm 1.1\%$ phospholipids, $5.1 \pm 0.7\%$ cholesterol esters, $9.1 \pm 1.2\%$ free cholesterol and $9.0 \pm 1.1\%$ protein. Composition of $\beta$-VLDL was $14.1 \pm 1.1\%$ triacylglycerols, $16.4 \pm 1.3\%$ phospholipids, $47.4 \pm 2.6\%$ cholesterol esters, $9.7 \pm 1.3\%$ free cholesterol and $10.6 \pm 0.6\%$ protein.

## RESULTS

### Effect of lactoferrin on serum decay and liver uptake of chylomicrons, $\beta$-VLDL and $\alpha_2$-macroglobulin

The liver uptake and serum decay of chylomicrons are shown in Figs. 1(*a*) and 1(*b*) respectively. In accordance with earlier data the serum decay follows a characteristic pattern which is explained by extrahepatic processing of chylomicrons to chylomicron remnants by endothelial cell-bound lipoprotein lipase with subsequent uptake by the liver (Redgrave, 1970). At 30 min after injection, 92% of the injected dose is cleared from the circulation with 84% of the injected dose recovered in the liver. Preinjection of 70 mg of lactoferrin/kg 1 min before injection of chylomicrons leads to a prolonged residence time of the chylomicron remnants as a consequence of a blockade of the liver uptake (at 5 min after injection only 9% of the injected dose is recovered in the liver as compared with 26% in the control). No significant difference in uptake by spleen and bone marrow was induced by lactoferrin (results not shown).

After injection of $^{125}$I-labelled $\beta$-VLDL into rats, the recognition by the liver is very fast. Preinjection of lactoferrin effectively blocks the liver uptake which leads to a prolonged residence time in the serum (Figs. 1*c* and 1*d*).

When $\alpha_2$-macroglobulin is treated with methylamine, it is converted into a substrate for the $\alpha_2$-macroglobulin receptor (Imber & Pizzo, 1981). In accordance with the data of Feldman *et al.* (1983) the serum decay of the so-treated $\alpha_2$-macroglobulin is very rapid ($t_{\frac{1}{2}}$ approx. 2 min) whereby at 10 min after injection 86% of the injected dose is recovered in the liver. At later time-points, radioactivity in the liver decreases probably as a consequence of degradation, as was also observed by Davidson *et al.*

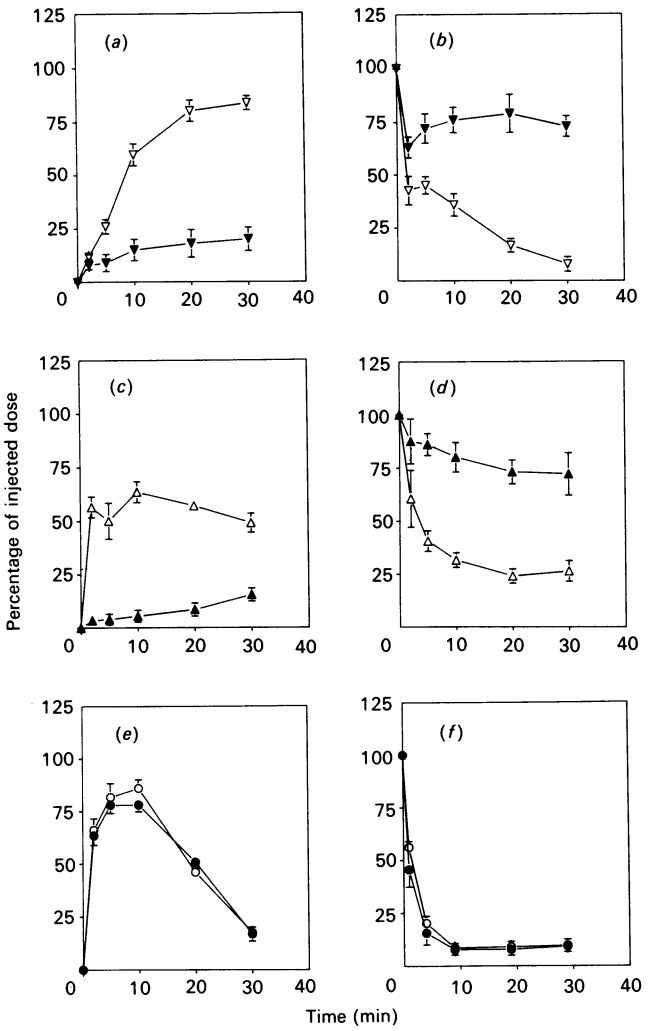


**Fig. 1. Effect of lactoferrin on liver uptake and serum decay of chylomicrons, β-VLDL and $\alpha_2$-macroglobulin**

$^3$H-labelled vitamin A-labelled chylomicrons (▽▼), $^{125}$I-labelled β-VLDL (△▲) or $^{125}$I-labelled $\alpha_2$-macroglobulin (○●) were injected into anaesthetized rats. Liver uptake (*a*, *c*, *e*) and serum decay (*b*, *d*, *f*) were determined with (closed symbols) and without (open symbols) prior injection of lactoferrin. Liver values are corrected for serum radioactivity. Values are means ± S.E.M. for at least three rats.

(1985) for the $\alpha_2$-macroglobulin–trypsin complex. Similar kinetics have previously been reported, i.e. for asialofetuin (Ashwell & Morell, 1974) and tissue-type plasminogen activator (Kuiper *et al.*, 1988). Preinjection of lactoferrin has no significant effect on the liver uptake and serum decay of $^{125}$I-labelled $\alpha_2$-macroglobulin (Figs. 1*e* and 1*f*).

### Cellular distribution of chylomicrons, β-VLDL and human $\alpha_2$-macroglobulin uptake

In order to analyse the cellular localization of the recognition sites for chylomicron remnants, β-VLDL and human $\alpha_2$-macroglobulin, liver parenchymal, endothelial and Kupffer cells were isolated by a low-temperature procedure at 15 min (chylomicrons and β-VLDL) or 8 min ($\alpha_2$-macroglobulin) after injection of radiolabelled (lipo)proteins. The various cell types were also isolated after preinjection of lactoferrin.

For chylomicrons and β-VLDL, the cellular distribution of the radioactivity was comparable. It appears that in Kupffer cells as compared with parenchymal cells, the percentage of injected dose per mg of cell protein is 2–4-fold higher, while the endothelial cell-associated radioactivity is either comparable with that of parenchymal cells or less. By taking into account the abundance of parenchymal cell protein in liver (92.5 % of total liver protein), it can be calculated (Table 1) that, for both substrates, the parenchymal cells are mainly responsible for the rapid hepatic clearance of chylomicron remnants (88.9 %) and β-VLDL (95.7 %). Preinjection of lactoferrin specifically blocks the recognition of chylomicron remnants and β-VLDL by parenchymal cells (to 13 % and 23 % respectively of the control value) leaving the uptake by endothelial or Kupffer cells virtually unaffected (Figs. 2*a* and 2*b*).

**Table 1. Relative contribution of the different liver cell types to the uptake of $^3$H-labelled vitamin A-labelled chylomicrons, $^{125}$I-labelled β-VLDL and $^{125}$I-labelled $\alpha_2$-macroglobulin by rat liver**

Different liver cell types were isolated 15 min (chylomicrons and β-VLDL) or 8 min ($\alpha_2$-macroglobulin) after injection. The amount of radioactivity/mg of cell protein in each cell fraction isolated was multiplied by the relative contribution of each cell type to total liver protein. Values are means ± S.E.M. for three experiments.

| Cell type | Chylomicrons | β-VLDL | $\alpha_2$-Macroglobulin |
|---|---|---|---|
| Parenchymal (%) | 88.9 ± 1.5 | 95.7 ± 0.7 | 44.8 ± 6.0 |
| Endothelial (%) | 2.7 ± 0.6 | 0.5 ± 0.2 | 42.8 ± 5.0 |
| Kupffer (%) | 8.4 ± 1.0 | 3.7 ± 0.7 | 12.6 ± 1.1 |

The uptake of $\alpha_2$-macroglobulin by the liver is carried out in a more even way by the various cell types. Endothelial liver cells show the highest percentage of the injected dose/mg of cell protein leading, after taking into account their relatively low protein contribution to total liver (3.3 %), to an equal contribution of parenchymal and endothelial cells to total liver uptake (Table 1). Preinjection of lactoferrin does not affect the uptake of $\alpha_2$-macroglobulin by any of the cell types (Fig. 2*c*).

### Specific effect of lactoferrin on the remnant receptor versus the LDL receptor

To identify the receptor recognition system that is specifically influenced by lactoferrin, we analysed the effect of lactoferrin under conditions in which LDL receptors are up-regulated. Recognition of β-VLDL can then be observed by both the LDL receptor and the remnant receptor. Oestradiol treatment of rats results in a 17-fold increase in LDL receptors (Chao *et al.*, 1979; Kovanen *et al.*, 1979), specifically on rat parenchymal cells (Harkes & van Berkel, 1983).

In oestradiol-treated rats, the serum decay of β-VLDL is faster than in control rats, while liver uptake of β-VLDL is significantly higher at 10, 20 and 30 min after injection (Figs. 3*a* and 3*b*). Under these conditions the effect of lactoferrin on the liver uptake and serum decay of β-VLDL is clearly less pronounced than in control rats. The inhibition of liver uptake by lactoferrin in oestradiol-treated rats is 35 % while this value is 92 % for control rats (at 5 min after injection).

Injection of $^{125}$I-labelled LDL into oestradiol-treated rats leads to rapid serum decay whereby at 5 min after injection 17.0 ± 4.0 % of the injected dose of LDL is associated with the liver (control 2.1 ± 0.2 %). As shown in Fig. 4, lactoferrin has no effect on the uptake of $^{125}$I-labelled LDL in oestradiol-treated rats. A direct comparison of the influence of lactoferrin on the initial liver uptake (determined 5 min after injection) illustrates the specificity of the effect of lactoferrin on uptake of chylomicron remnants and β-VLDL in control rats (Fig. 4).

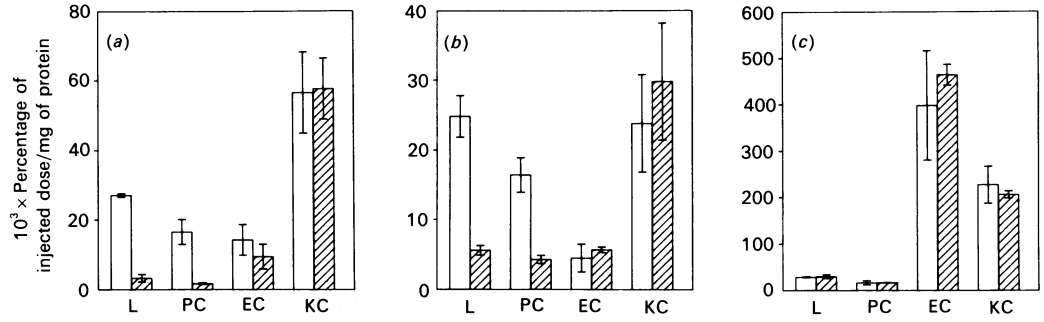


**Fig. 2. Effect of lactoferrin on the association *in vivo* of chylomicrons, β-VLDL and $\alpha_2$-macroglobulin with parenchymal, endothelial and Kupffer cells**

$^3$H-labelled vitamin A-labelled chylomicrons (*a*), $^{125}$I-labelled β-VLDL (*b*) or $^{125}$I-labelled $\alpha_2$-macroglobulin (*c*) were injected into anaesthetized rats with (hatched bars) or without (open bars) prior injection of lactoferrin. At 15 min (*a*+*b*) or 8 min (*c*) after injection, the liver was perfused at 8 °C. After 8 min of perfusion, total liver association of radioactive lipoproteins was determined (L) and subsequently parenchymal (PC), endothelial (EC) and Kupffer (KC) cells were isolated by a low-temperature method. Values are means ± S.E.M. for three rats. For chylomicrons and β-VLDL, values for parenchymal cells with lactoferrin are significantly ($P < 0.05$) different from those without.

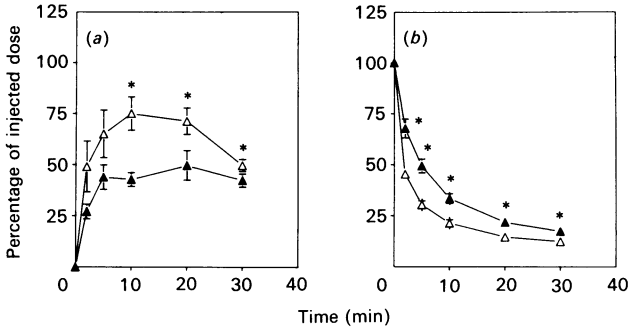


**Fig. 3. Effect of lactoferrin on liver uptake and serum decay of β-VLDL in 17α-ethinyloestradiol-treated rats**

Rats were treated with 17α-ethinyloestradiol (5 mg/kg) for 3 days and injected with $^{125}$I-labelled β-VLDL (△▲). Liver uptake (*a*) and serum decay (*b*) were determined with (closed symbols) and without (open symbols) prior injection of lactoferrin. Liver values are corrected for serum radioactivity. Values are means ± S.E.M. for three rats. * Significantly different ($P < 0.05$) from controls.

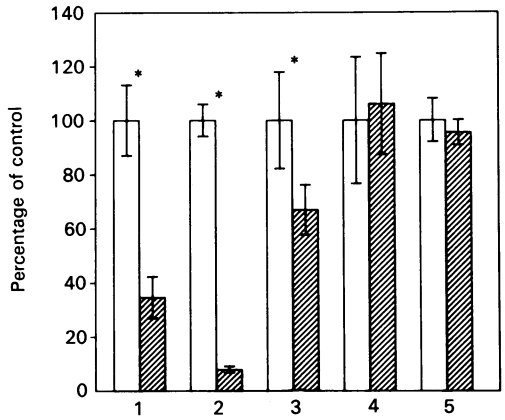


**Fig. 4. Effect of lactoferrin on uptake of (lipo)proteins by the liver**

Rats were injected with radiolabelled (lipo)proteins and 5 min after injection uptake by the liver, with (hatched bars) and without (open bars) prior injection of lactoferrin, was determined. Values are means ± S.E.M. for three rats. * Significantly different ($P < 0.05$). 1, Uptake of chylomicrons; 2, uptake of β-VLDL; 3, uptake of β-VLDL in oestradiol-treated rats; 4; uptake of LDL in oestradiol-treated rats; 5, uptake of $\alpha_2$-macroglobulin.

### Nature of recognition site for chylomicron remnants and β-VLDL on rat parenchymal cells

Earlier studies have revealed that β-VLDL can be recognized by both the LDL and remnant receptor (R. De Water, J. A. A. M. Kamps, M. C. M. van Dijk, E. M. A. J. Hessels, J. Kuiper, J. K. Kruijt & Th. J. C. van Berkel, unpublished work). For the reason that parenchymal cells from control rats hardly expressed LDL receptors at all (Harkes *et al.*, 1984*a*,*b*), it was previously concluded that the remnant receptor is responsible for the interaction of β-VLDL with these cell types (Harkes *et al.*, 1989). The present competition experiments provide direct evidence that β-VLDL and chylomicron remnants do compete for the same recognition site (Figs. 5*a* and 5*b*). The experiments were performed at 37 °C instead of 4 °C, because the use of a physiological temperature is important for optimal lipoprotein–receptor interaction (Cooper *et al.*, 1982). A possible explanation for the temperature-dependence of chylomicron remnant binding to rat parenchymal cells *in vitro* may be a non-optimal conformation of apoE as ligand for the remnant receptor as a consequence of the altered fluidity of shell phospholipids at low temperatures. It appears that β-VLDL is a very effective competitor for $^{125}$I-labelled chylomicron remnant association to parenchymal cells. The remnants themselves are also effective competitors, whereas LDL is essentially ineffective. Methylamine-activated $\alpha_2$-macroglobulin does not compete with $^{125}$I-labelled chylomicron remnants for cell association.

With radioiodinated β-VLDL as labelled ligand, similar competition curves were obtained to those with radioactive chylomicron remnants, indicating that chylomicron remnants and β-VLDL compete for the same recognition site, whereas no competition is observed with LDL or $\alpha_2$-macroglobulin (Fig. 5*b*).

In order to verify that the isolated parenchymal cells do express the specific high-affinity site for $\alpha_2$-macroglobulin, the cells were incubated with $^{125}$I-labelled $\alpha_2$-macroglobulin in the absence or presence of increasing concentrations of unlabelled $\alpha_2$-macroglobulin (Fig. 5*c*). It appears that unlabelled $\alpha_2$-macroglobulin competes effectively with $^{125}$I-labelled $\alpha_2$-macroglobulin for cell association whereas both LDL and β-VLDL are ineffective.

### Regulation of the recognition sites for β-VLDL and $\alpha_2$-macroglobulin of cultured rat parenchymal cells

The expression of receptors on liver parenchymal cells in culture is subject to regulatory changes, i.e. the asialofetuin

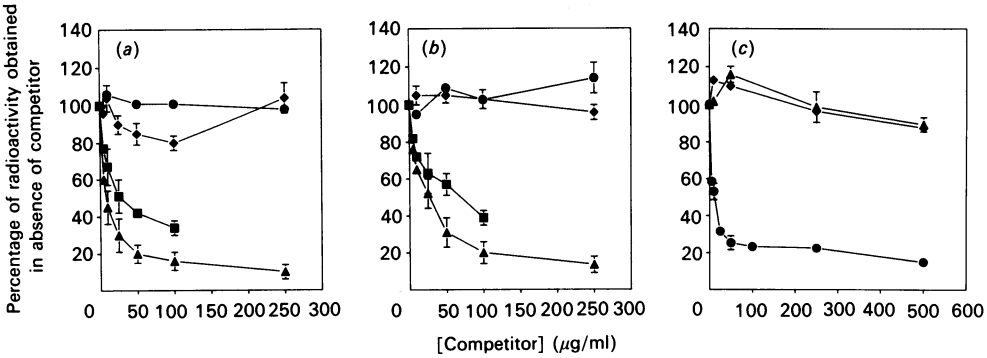


**Fig. 5. Ability of unlabelled lipoproteins to compete with $^{125}$I-labelled chylomicron remnants, $^{125}$I-labelled β-VLDL and $^{125}$I-labelled $\alpha_2$-macroglobulin for association with rat parenchymal cells**

Freshly isolated rat parenchymal cells were incubated for 10 min at 37 °C with 5 μg of apolipoprotein/ml of $^{125}$I-labelled chylomicron remnants (*a*), 5 μg of apolipoprotein/ml of $^{125}$I-labelled β-VLDL (*b*) or 3.6 nM-$^{125}$I-labelled $\alpha_2$-macroglobulin (*c*) in the absence or presence of unlabelled chylomicron remnants (■), β-VLDL (▲), LDL (◆) or $\alpha_2$-macroglobulin (●). The binding is expressed as the percentage of the radioactivity obtained in the absence of competitor. The 100% binding values for $^{125}$I-labelled chylomicron remnants, $^{125}$I-labelled β-VLDL and $^{125}$I-labelled $\alpha_2$-macroglobulin respectively were 93.3±12.6, 88.7±13.3 and 28.0±4.0 ng of protein/mg of cell protein. Values are means±S.E.M. of three experiments.

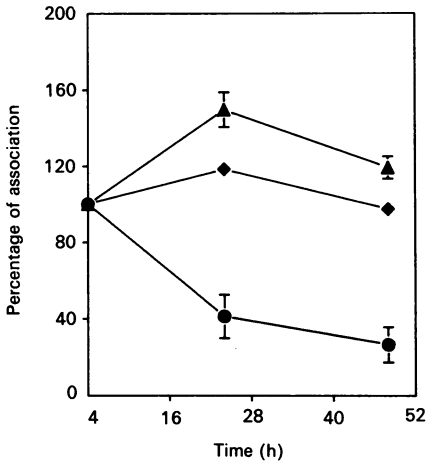


**Fig. 6. Cell association of $^{125}$I-labelled β-VLDL and $^{125}$I-labelled $\alpha_2$-macroglobulin with rat parenchymal cells in culture**

Rat parenchymal cells were cultured at 37 °C. At 4, 24 and 48 h the cell association of $^{125}$I-labelled β-VLDL (10 μg of apolipoprotein/ml) in the absence (▲) or presence (◆) of an excess (300 μg of apolipoprotein/ml) of unlabelled human LDL was determined. Simultaneously the cell association of $^{125}$I-labelled $\alpha_2$-macroglobulin (3.6 nM) (●) was analysed. The 4 h values were taken as 100%. Values are means±S.E.M. of four experiments. With excess LDL, values are means of two experiments.

receptor is diminished during the first 24 h of culture (Drevon *et al.*, 1983). Feldman *et al.* (1985) reported that rat hepatocytes also exhibited a significant decrease in $\alpha_2$-macroglobulin receptor number during primary culture. A direct comparison of the regulation of the remnant and $\alpha_2$-macroglobulin receptor in culture (Fig. 6) indicates that the cell association of $\alpha_2$-macroglobulin diminishes rapidly to 26% of the initial value after 48 h of culture. In contrast the cell association of $^{125}$I-labelled β-VLDL shows an increase. Because LDL receptors in particular are up-regulated in culture, we also determined the association of $^{125}$I-labelled β-LDL in the presence of an excess of unlabelled LDL in order to correct for the LDL-receptor-dependent interaction of β-VLDL. It is clear that the expression of the remnant receptor (measured in the presence of an excess of unlabelled LDL) does not decrease with parenchymal cells in culture.

## DISCUSSION

The existence of a remnant receptor in liver is suppported by observations in patients with homozygous familial hypercholesterolaemia and WHHL rabbits in whom LDL receptors are genetically defective (Kita *et al.*, 1982; Hoeg *et al.*, 1985). Both chylomicron remnants and large VLDL particles are rapidly cleared from the blood in these LDL-receptor-defective species, suggesting that a second (remnant) receptor may mediate such a clearance. Also, intravenous infusion of apoE into cholesterol-fed normal rabbits, who have down-regulated their LDL receptors, lowers plasma cholesterol concentration, suggesting that apoE may mediate the uptake of lipoproteins through pathways independent of the LDL receptor (Mahley *et al.*, 1989). Recently we performed a characterization of the interaction of β-VLDL with rat parenchymal cells, leading to the conclusion that β-VLDL in normal rats does not interact with the LDL receptor but is primarily a substrate for the remnant receptor (R. De Water, J. A. A. M. Kamps, M. C. M. van Dijk, E. M. A. Hessels, J. Kuiper, J. K. Kruijt & Th. J. C. van Berkel, unpublished work). The present data provide conclusive evidence for a mutual competition between β-VLDL and chylomicron remnants for the remnant receptor on parenchymal cells whereas LDL does not compete at all. Parenchymal cells from untreated rats (Harkes & van Berkel, 1984*a*), in contrast with cells from rabbits (Nenseter *et al.*, 1988) or human parenchymal cells (Kleinherenbrink-Stins *et al.*, 1991), contain very few LDL receptors, which explains the total absence of competition by LDL. This conclusion is based on work with human LDL because this preparation can be obtained completely free of apoE (Harkes & van Berkel, 1984*a*), which is important to prevent any interaction with the remnant receptor. Human LDL is readily recognized by oestrogen-induced LDL receptors in rats, so that an absence of competition cannot be explained by interspecies variation of LDL-receptor recognition. The clearance from the blood and liver association of β-VLDL and chylomicron remnants *in vivo* is blocked by lactoferrin. When LDL receptors in rats are up-regulated *in vivo* by oestrogen treatment, it appears that lactoferrin is much less effective, which indicates that lactoferrin does not interfere with the binding of β-

VLDL to the LDL receptor. Under these conditions the uptake of human LDL by liver is stimulated 8-fold, which indicates that human LDL is readily recognized by the induced LDL receptors and lactoferrin is totally ineffective in modifying this induced uptake.

Herz *et al.* (1988) suggested that a high-$M_r$ protein (the LRP) might be an attractive candidate to function as the remnant receptor. Subsequently Kowal *et al.* (1989, 1990), Lund *et al.* (1989) and Beisiegel *et al.* (1989) provided evidence that apoE might bind to LRP, although enrichment *in vitro* of $\beta$-VLDL with apoE was necessary to express binding. We showed earlier that the kinetics of binding of $\beta$-VLDL to parenchymal cells *in vivo* (Harkes *et al.*, 1989) and the properties of cell association *in vitro* do not indicate that additional apoE is needed for optimal interaction of $\beta$-VLDL with the remnant receptor on parenchymal cells. The debate over whether the remnant receptor is LRP has recently been intensified by the finding that the $\alpha_2$-macroglobulin receptor and LRP are the same molecule. Ten peptides derived from the $\alpha_2$-macroglobulin receptor by chemical or proteolytic digestion were sequenced and all appeared to be present within the cDNA-deduced structure of LRP (Strickland *et al.*, 1990). It is speculated that LRP is a multifunctional receptor with the capacity to bind diverse biological ligands. The present direct comparison of the characteristics of the $\alpha_2$-macroglobulin receptor and the remnant receptor *in vivo* and *in vitro* does not confirm this speculation. The following properties point to different sites for chylomicron remnants/$\beta$-VLDL and $\alpha_2$-macroglobulin recognition.

1. *In vivo*, lactoferrin blocks specifically chylomicron remnant and $\beta$-VLDL uptake by parenchymal cells, leaving $\alpha_2$-macroglobulin uptake unaffected.

2. Competition studies *in vitro* indicate that chylomicron remnants and $\beta$-VLDL compete for the same recognition site whereas no mutual competition with the high-affinity site for $\alpha_2$-macroglobulin is observed.

Evidence that the recognition sites for chylomicron remnants/$\beta$-VLDL and $\alpha_2$-macroglobulin reside on different molecules can be deduced from the following additional findings.

3. Recognition of the chylomicron/$\beta$-VLDL particles is mainly by parenchymal cells whereas recognition of $\alpha_2$-macroglobulin is shared between parenchymal and endothelial cells.

4. A primary culture of liver parenchymal cells showed a significant decrease in $\alpha_2$-macroglobulin receptors whereas the recognition of $\beta$-VLDL by the remnant receptor is not influenced.

These four considerations provide conclusive evidence that the recognition sites for chylomicron remnants/$\beta$-VLDL and $\alpha_2$-macroglobulin may reside in different parts of the receptor. Points 3 and 4 indicate that these sites reside on different molecules. As mentioned earlier, the tissue distribution of LRP does not coincide with the sites of chylomicron remnant/$\beta$-VLDL uptake, as LRP is found nearly ubiquitously throughout animal cells (Herz *et al.*, 1988). Furthermore, for LRP, it was reported that $Ca^{2+}$ is obligatory for binding of apoE-enriched $\beta$-VLDL (Kowal *et al.*, 1989). Similar results concerning $Ca^{2+}$ were reported for the ligand recognition of the $\alpha_2$-macroglobulin receptor (Moestrup *et al.*, 1990). The binding of $\beta$-VLDL to parenchymal cells is, however, independent of added $Ca^{2+}$ (R. De Water, J. A. A. M. Kamps, M. C. M. van Dijk, E. M. A. Hessels, J. Kuiper, J. K. Kruijt & Th. J. C. van Berkel, unpublished work). When these additional two points are added to the four considerations mentioned above, we conclude that the remnant receptor of parenchymal cells, which recognizes both chylomicron remnants and $\beta$-VLDL, possesses distinct properties and may not be identical with LRP. This conclusion suggests the need for further work to characterize the molecular nature of the remnant receptor. The availability of a non-lipoprotein competitive ligand (lactoferrin) for the remnant receptor might provide an important additional experimental tool.

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