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Apoprotein-independent binding of chylomicron remnants to rat liver membranes

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Rat lymph chylomicrons and chylomicron remnants were treated with trypsin or Pronase. The ability of the resulting apoprotein-free lipoproteins to be taken up by the isolated perfused rat liver, and to bind to isolated rat liver membranes, was examined. Compared with control lipoproteins, the apoprotein-free chylomicrons and remnants retained unaltered their capacity to be differentiated by the intact liver and by the isolated membranes. Further, control remnants and apoprotein-free remnants competed for binding to the isolated membranes. We conclude that apoproteins are not required for the hepatic differentiation between chylomicrons and remnants, and suggest that the lipoprotein phospholipids may play a direct role in this process.

INTRODUCTION

The removal of chylomicrons from the circulation is a complex process that begins with their partial degradation in the vascular space, and ends with the uptake of the resulting particles, the chylomicron remnants, by the liver (Redgrave, 1970). There is considerable evidence that hepatic uptake of remnants occurs by a receptor-mediated process (Mahley *et al.*, 1989), but many aspects of this process remain to be fully elucidated. Specifically, the identity of the receptor, and the requisite compositional changes in the chylomicron which allow recognition of the resulting remnant by the receptor, are not yet established (Mahley *et al.*, 1989; Soutar, 1989). It is widely believed that remnant apoprotein E is the ligand for the hepatic receptor (Mahley *et al.*, 1989), but that effective ligand–receptor interaction does not occur until the apoprotein E/C ratio of the particle is sufficiently increased by the loss of apoprotein C during the chylomicron degradation (Windler *et al.*, 1980*a*,*b*; Shelburne *et al.*, 1980; Quarfordt *et al.*, 1982; Windler & Havel, 1985; Weisgraber *et al.*, 1990; Kowal *et al.*, 1990). Work from our laboratory has shown, however, that chylomicrons and remnants which have been modified so as to have similar apoprotein E/C ratios are still differentiated by the liver (Borensztajn & Kotlar, 1984).

In addition to the changes in relative apoprotein concentrations, the generation of remnants is also accompanied by modification in phospholipid composition (Redgrave & Small, 1979; Tall *et al.*, 1979; Landin & Nilsson, 1984). We have examined whether these phospholipid alterations, rather than changes in the apoprotein E/C ratio, might account for the hepatic differentiation of the lipoproteins. We have demonstrated that chylomicrons treated *in vitro* with phospholipase A_2 (Borensztajn *et al.*, 1980; Borensztajn & Kotlar, 1981) or with hepatic lipase (Borensztajn *et al.*, 1988), which causes little or no change in apoprotein composition, are transformed into 'remnants' that can be readily removed from circulation by the liver and can compete with endogenous remnants for uptake by hepatocytes (Borensztajn *et al.*, 1988). On the basis of these results, we have postulated that phospholipid changes during remnant formation promote hepatic uptake by modulating the apoprotein E interaction with the receptor. However, an alternative hypothesis is that phospholipids might determine the hepatic recognition of remnants independently of apoprotein E. As a first step in the investigation of this hypothesis, we examined whether chylomicron remnants devoid of apoprotein E can bind to isolated liver membranes. We report that the removal of apoprotein E, as well as all other apoproteins, from chylomicrons and remnants does not abolish the ability of liver membranes to differentiate between these particles. In addition, we report, on the basis of competition studies, that control and apoprotein-free remnants bind to the membranes by the same mechanism. The implications of these observations for current concepts of remnant recognition by the liver are discussed.

MATERIALS AND METHODS

Preparation of small chylomicrons

The thoracic ducts of male Sprague–Dawley rats (180–220 g) were cannulated by the technique of Bollman *et al.* (1948), and the animals were then fed by stomach intubation on a mixture of 1 ml of corn oil and 3 ml of egg yolk. In some experiments, 100 μCi of [1,2-^{3}H]cholesterol (sp. radioactivity 50 Ci/mol; Amersham Corp.) and 30 μCi of [1-^{14}C]palmitic acid (sp. radioactivity 58 Ci/mol; Amersham Corp.) were added to the mixture. The lymph was collected during the following 16 h at room temperature in the absence of preservatives. After declotting, the chylomicrons were centrifuged at 80000 *g* for 45 min at 15 °C in a Beckman model L5-75 ultracentrifuge, as previously described (Borensztajn *et al.*, 1985). After removal of the floating chylomicron layer, the remaining lipoproteins were spun further in the ultracentrifuge at 80000 *g* for 16 h at 15 °C and the floating small chylomicrons were harvested. Before being used for membrane-binding assays, the chylomicrons were then purified by gel-permeation chromatography as previously described (Borensztajn *et al.*, 1985).

Preparation of remnants

For the preparation of chylomicron remnants, post-heparin plasma was obtained by bleeding male Sprague–Dawley rats (350–450 g) 10 min after they had been injected intravenously with 60 i.u. of heparin/kg. The post-heparin plasma was collected after centrifugation of the blood at 2000 *g* for 20 min at 5 °C. The small chylomicrons were then incubated with the post-heparin plasma (3 mg of triacylglycerols/ml) for 4 h at 37 °C. The density of the incubation mixture was then adjusted to 1.019 g/ml by addition of NaCl. This incubation mixture was then layered under an equal volume of an NaCl solution of density 1.019 g/ml and spun at 80000 *g* for 18 h at 15 °C in a Beckman model L5-75 ultracentrifuge in a SW 27 rotor. The

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floating layer of remnants was harvested, and purified by gel-permeation chromatography as previously described (Borensztajn *et al.*, 1985). With this procedure, 70–80% of the chylomicron triacylglycerols were hydrolysed.

Liver perfusion

The technique for liver perfusion was essentially as previously described (Borensztajn & Kotlar, 1984). The basic perfusion medium was Krebs–Ringer bicarbonate buffer (pH 7.4), continuously gassed with O_2/CO_2 (19:1), containing 3% (w/v) BSA (Sigma) and 1 mg of glucose/ml. Chylomicrons or remnants were added to 30 ml of this medium at a concentration of 9 μg of cholesterol/ml. Livers were first perfused without recirculation for 5 min with the basic medium to remove blood from the preparation. This was followed by a 4 min recirculatory perfusion with the various lipoprotein preparations, and finally a 2 min perfusion without recirculation with the basic medium to wash out lipoproteins that might have been trapped in the sinusoidal spaces. After the washing, the livers were blotted and weighed, and 1 g samples were taken for radioactivity measurement as previously described (Borensztajn & Kotlar, 1984). All livers were perfused at a flow rate of 14 ml/min.

Membrane-binding assay

Rat liver plasma membranes were isolated by the method of Windler *et al.* (1980*c*). Binding assay was carried out by incubating lipoproteins with plasma membranes in buffer containing 50 mM-NaCl, 1 mM-$CaCl_2$ and 20 mM-Tris/HCl, pH 7.5, to a final volume of 0.5 ml in 1.5 ml microfuge tubes. Each assay contained 100 μg of membrane protein, and blanks containing no membrane were also included in each experiment. Preliminary experiments showed that lipoprotein binding increased directly with the quantity of membrane in the assay. For all lipoproteins the blanks accounted for no more than 0.3 μg of cholesterol. Incubation was carried out at 37 °C for 60 min, and bound lipoproteins were separated from the unbound by centrifuging in a Brinkmann Microcentrifuge at 16000 *g* for 20 min. The supernatant was aspirated and replaced with fresh buffer, and re-centrifuged under the same conditions. The supernatant was then discarded, and the bottom of the tube containing the membrane pellet was cut into a counting vial containing 5 ml of scintillation fluid. The vial was then sonicated for 30 s, at 70% efficiency and output set at 8, with a Heat Systems cup horn sonicator, model 350 (Heat Systems Ultrasonics, Farmingdale, NY, U.S.A.) to disperse the pellet before radioactivity measurement.

Competition was carried out by adding 100 μg of membrane protein to 1.5 ml polyallomer tubes (Beckman) containing labelled remnants and buffer. Unlabelled remnants were added 2 min later and the mixture was incubated at 37 °C for 60 min. The final volume of each assay was 0.5 ml. Blanks containing no membranes were also included in all experiments. After incubation, the tubes were centrifuged in a TL-100 ultracentrifuge (Beckman), in a TLA-45 rotor, at 125000 *g* for 20 min at 25 °C. The supernatant was replaced with fresh buffer and the tubes were re-centrifuged under the above conditions. After centrifugation, the supernatant was discarded and the bottom of the tube was cut off into a counting vial containing 5 ml of scintillation fluid. The vials were then sonicated, as described above, and counted for radioactivity.

Other procedures

Treatments of chylomicrons and remnants with trypsin (Sigma) or Pronase (Sigma) were as previously described (Borensztajn *et al.*, 1982; Borensztajn & Kotlar, 1984). The complete digestion of the apoproteins was verified by SDS/PAGE. After treatment with the proteinases, the lipoproteins were re-purified by centrifugation and gel permeation (Borensztajn *et al.*, 1982; Borensztajn & Kotlar, 1984). Lipoprotein cholesterol was measured by the method of Abell *et al.* (1952), and triacylglycerols were measured by the method of Bucolo & David (1973) with a Synchron CX Analyzer (Beckman Instruments).

RESULTS AND DISCUSSION

Previous work from this laboratory (Borensztajn & Kotlar, 1984) has shown that chylomicron remnants generated *in vivo*, completely stripped of their surface apoproteins by proteinase treatment and subsequently incubated with serum, re-acquired all their water-soluble apoproteins, including apoprotein E. The mass of apoprotein E on the reconstituted remnants, however, was considerably less than that of the untreated remnants, as determined by SDS/PAGE. Nevertheless, this decrease in apoprotein E content of the treated remnants did not decrease their rate of uptake by the isolated perfused rat liver. In the present study we set out to investigate whether remnants altogether devoid of apoprotein E could be recognized and taken up by the liver. For this purpose, [^{14}C]fatty acids/[^{3}H]cholesterol-labelled chylomicrons and chylomicron remnants that had been treated with trypsin, but without reconstitution by incubation with serum, were perfused through isolated rat liver preparations as described in the Materials and methods section. The results obtained (Table 1) show that the ability of the liver to differentiate chylomicrons and remnants was not impaired by the removal of all apoproteins from the surface of the particles. The 4.5-fold difference in uptake between apoprotein-free chylomicrons and apoprotein-free remnants was similar to that of their respective controls. Comparable results were observed when the uptake was calculated as % of [^{14}C]fatty acids in the perfusate taken up by the liver (results not shown). These results suggested that apoprotein E may not be required for the hepatic recognition of remnants. Since in the isolated perfused liver lipoproteins and, presumably, also free apoprotein E are continuously secreted by the hepatocytes into the perfusion medium (Marsh, 1986), the possibility could not be ruled out that the proteinase-treated remnants re-acquired apoprotein E before being taken up by the hepatocytes. However, for this explanation to be valid, it is necessary to postulate either that the proteinase-treated chylomicrons, which were poorly taken up by the liver, did not acquire apoprotein E, or that they acquired lesser amounts of this apoprotein than the proteinase-treated remnants. An alternative explanation is that, unlike remnants, the proteinase-treated chylomicrons acquired apoprotein E in a conformation that does not allow its recognition by the receptor.

To avoid these problems of potential apoprotein E contamination of the lipoproteins in the intact liver, we examined the ability of control and apoprotein-free chylomicrons and remnants to bind to isolated liver membranes, an experimental system that has been successfully used in the investigation of lipoprotein receptors (Carella & Cooper, 1979; Cooper *et al.*, 1982; Kita *et al.*, 1982; Hui *et al.*, 1984; Windler *et al.*, 1988; Nagata *et al.*, 1988). Fig. 1(*a*) shows the results obtained when control chylomicrons and remnants were incubated with the isolated liver membranes. In agreement with previous observations (Carella & Cooper, 1979; Cooper *et al.*, 1982; Hui *et al.*, 1984; Windler *et al.*, 1988), only remnants bound efficiently to the isolated membranes. Binding occurred in a saturable manner, with saturation observed at a concentration of 37 μg of cholesterol/mg of membrane protein. Fig. 1(*b*) shows the results obtained when chylomicrons and remnants that had been pre-treated with trypsin were incubated with the isolated membranes. It is apparent that the apoprotein-free remnants retained unchanged

Table 1. Uptake of control and trypsin-treated chylomicrons and remnants by isolated perfused rat livers

Livers (4 per group) were first perfused in a non-recirculatory system to wash out trapped blood, and then perfused with the labelled lipoproteins (9 μg of lipoprotein cholesterol/ml) for 4 min, as described in the Materials and methods section. The results are expressed as percentage of lipoprotein cholesterol in perfusate taken up/liver (mean $\pm$ S.D.).

	Control lipoproteins	Trypsin-treated lipoproteins
Chylomicrons	4.8 $\pm$ 0.8	4.5 $\pm$ 0.7
Remnants	21.4 $\pm$ 2.0	23.6 $\pm$ 3.1

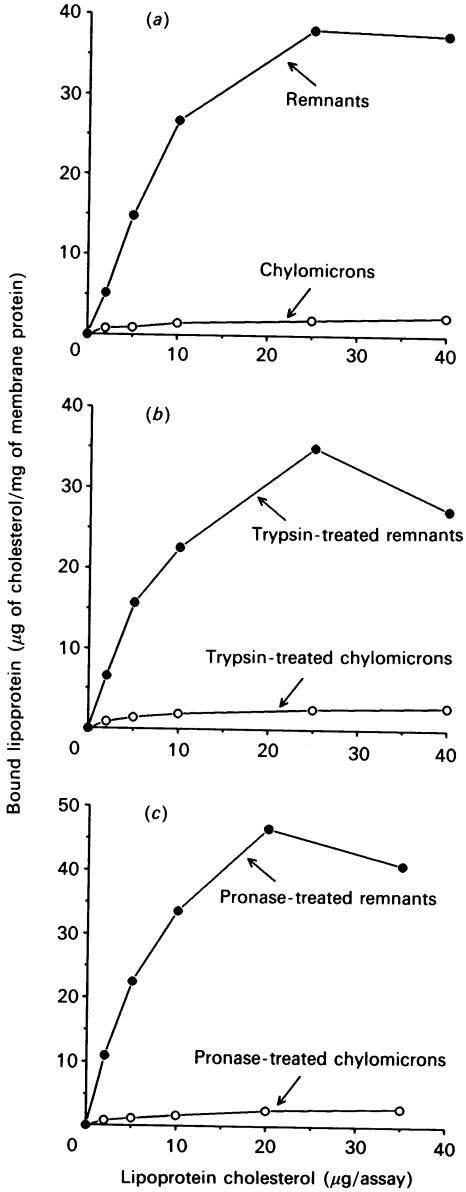

Fig. 1. Binding of proteinase-treated chylomicrons and remnants to rat liver membranes

[^{14}C]Palmitic acid/[^{3}H]cholesterol-labelled control chylomicrons and remnants (*a*), or chylomicrons and remnants made apoprotein-free by trypsin (*b*) or Pronase (*c*) treatment, were incubated with 100 μg of membrane protein in the indicated amounts, as described in the Materials and methods section. Each point represents the average of triplicate assays.

their ability to bind to the isolated membranes. Under the conditions used, all apoproteins from the chylomicrons and the remnants, including apoprotein E, were digested by the proteinase, as previously reported (Borensztajn & Kotlar, 1984). It is possible that, after trypsin digestion, apoprotein E fragments remained attached to the surface of the lipoproteins. It is unlikely, however, that such fragments would mediate the binding of remnants to the membranes or, in that eventuality, that they would do so as efficiently as the intact apoprotein. Bradley & Gianturco (1986) reported that trypsin treatment of very-low-density lipoproteins caused the degradation of their apoprotein E and, as a result, the complete loss of their ability to bind to fibroblast receptors. Bates *et al.* (1987) reported that trypsin digestion of apoprotein E on the surface of β-very-low-density lipoproteins (β-VLDL) abolished the ability of these lipoprotein remnants to be recognized by receptors on the surface of macrophages. Connelly *et al.* (1988) reported that treatment of β-VLDL with trypsin abolished its ability to displace low-density lipoprotein from fibroblasts.

To exclude further the possibility that apoprotein E fragments might have mediated the binding of the remnants to the membranes, we repeated the binding experiments, using remnants made apoprotein-free by treatment with Pronase. The use of Pronase, a mixture of proteolytic enzymes isolated from *Streptomyces griseus*, ensured a more extensive digestion of the apoproteins. In a previous report, no apoprotein B fragments were detected on the surface of chylomicrons after their treatment with Pronase (Borensztajn *et al.*, 1982). The results in Fig. 1(*c*) show that, like the intact (Fig. 1*a*) or the trypsin-treated (Fig. 1*b*) remnants, the Pronase-treated particles retained unchanged their ability to bind to the membranes. If binding were mediated by fragments of apoprotein E generated by the mixture of non-specific proteinases (Pronase), it would be necessary to postulate (*a*) that such fragments resembled those generated by trypsin, and (*b*) that they retained fully the binding ability of intact apoprotein E.

Because the remnants were double-labelled with [^{14}C]fatty acids and [^{3}H]cholesterol, it was possible to determine that in all cases the remnants bound as a unit to the membranes. In the experiment shown in Fig. 1(*c*), the ^{14}C/^{3}H ratio of the remnants added to the isolated membranes was 0.11, and the ratio of the radioactivity recovered associated with the membranes was 0.10 $\pm$ 0.01 (mean $\pm$ S.D., $n = 15$). To determine whether the apoprotein-free and control remnants shared the same mechanism of binding to the membranes, we compared the ability of unlabelled control and trypsin-treated remnants to compete with [^{3}H]cholesterol-labelled remnants for binding to the membranes. Fig. 2 shows that both types of particles effectively displaced the binding of the labelled remnants. Although only a 60% displacement was observed with an 8-fold excess of unlabelled remnants, the curves in Fig. 1 indicate that little, if any, remnant binding is non-specific. The competition curve, if extended to a sufficiently high excess of unlabelled remnants, would presumably demonstrate the same. Such an experiment was not feasible, however. The viscosity of the binding assay with very high concentrations of lipoproteins apparently interferes with normal remnant binding. Thus at an excess of only 20-fold, not only remnants, but also chylomicrons (which do not bind to the membranes; Fig. 1), cause an 'inhibition' of labelled-remnant binding.

The results described above, demonstrating that apoprotein-free remnants retain intact their ability to be cleared by the intact liver and to bind to liver membranes, may appear to be at variance with other reports which also examined how the depletion of apoprotein E might affect the recognition of remnants by the liver. For example, Hui *et al.* (1984) reported

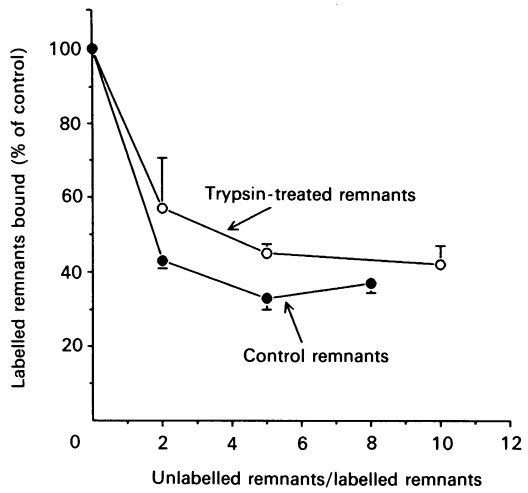

Fig. 2. Specificity of remnant and apoprotein-free remnant binding

[^{14}C]Palmitic acid/[^{3}H]cholesterol-labelled remnants (25 μg of cholesterol) were added to 100 μg of membrane protein, followed by the indicated excess of unlabelled control remnants or trypsin-treated remnants. The incubation conditions were as described in the Materials and methods section. Each point shows the mean $\pm$ S.D. ($n = 3$).

that apoprotein E-deficient remnants did not bind to receptors on isolated dog liver membranes, whereas chylomicron remnants containing this apoprotein bound with high affinity. It is noteworthy, however, that in that study (Hui *et al.*, 1984) the control and apoprotein E-deficient remnants were prepared by different methods, and, although the differences in their binding to the membranes were attributed solely to differences in their apoprotein E content, the particles also differed significantly in their lipid composition. For example, the phospholipid/triacylglycerol ratio of the control remnants was 0.26, whereas in the remnants deficient in apoprotein E this ratio was 0.44. These differences in phospholipid content of the remnants are pertinent, in light of the proposition that phospholipids play a major role in the hepatic uptake of remnants (Borensztajn & Kotlar, 1990; see also the Introduction). In another study, Arbeeny & Rifici (1984) reported that remnants deficient in apoprotein E were cleared by the isolated perfused liver less efficiently than control remnants. However, in that study the comparison was also carried out between control and apoprotein E-deficient remnants that had been prepared by quite different methods. Whereas apoprotein E-rich remnants were prepared from chylomicrons injected into normal hepatectomized rats, the apoprotein E-deficient remnants were prepared by using hepatectomized ethinyloestradiol-treated rats injected with chylomicrons obtained from animals that had also been treated with that sterol. It is known that the pharmacological dose of ethinyloestradiol used in that study has significant effects in the overall composition of plasma lipoproteins, including chylomicrons (Davis & Roheim, 1978; Chao *et al.*, 1979; Krause *et al.*, 1981). It is therefore conceivable that the reported differences in the hepatic clearance of remnants (Arbeeny & Rifici, 1984) might be explained by factors other than their apoprotein E content.

A role for apoprotein E in the hepatic recognition of remnants has also been proposed, on the strength of observations that addition of this apoprotein to lipoproteins and lipid emulsions stimulates the hepatic clearance of these particles (Shelburne *et al.*, 1980; Windler & Havel, 1985). Several proteins capable of binding apoprotein E have been described in a variety of cells. One such protein found in liver cell membranes, the low-density-lipoprotein-receptor-related protein (LRP), has been shown to bind cholesterol-rich remnants (β-VLDL) *in vitro*, and it has been proposed to function as the hepatic remnant receptor *in vivo* (Beisiegel *et al.*, 1989; Kowal *et al.*, 1990; Weisgraber *et al.*, 1990). It is noteworthy, however, that LRP binds β-VLDL only when an excess of apoprotein E is added to these lipoproteins. It is conceivable that LRP, or other apoprotein E-binding proteins, may facilitate the hepatic clearance of lipoproteins and emulsions artificially enriched with apoprotein E (Shelburne *et al.*, 1980; Windler & Havel, 1985). However, the present results with the isolated perfused rat liver and isolated liver membranes suggest that the hepatic clearance of remnants from circulation may occur by an apoprotein E-independent mechanism. We have previously demonstrated that phospholipids play an important role in the uptake of remnants by the liver, and proposed that they function by modulating the binding of apoprotein E to the putative remnant receptor (Borensztajn & Kotlar, 1984, 1990; Borensztajn *et al.*, 1988). In light of the present study, it is possible that phospholipids function as main determinants of remnant recognition by the liver, independently of apoprotein E.

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