Forces involved in chylomicron binding by isolated cells of rat liver

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ABSTRACT The binding of chylomicrons by isolated liver cells has been found to decrease as temperature increases. It is greatest at the isoelectric point of the chylomicrons; although it occurs both above and below this pH, it decreases most rapidly as the pH is increased.

Urea, guanidine hydrochloride, dimethylsulfoxide, dioxane, and sodium chloride at concentrations known to disrupt bonding in proteins have no effect on the removal (by centrifugation) of chylomicrons bound to liver cells.

The binding is reduced by treatment of chylomicrons with phospholipase D or by addition of chylomicron "membrane" fraction, lecithin micelles, or lecithin–triglyceride–cholesterol micelles. This evidence implicates phospholipids in the binding.

Treatment of liver cells with neuraminidase increases binding of chylomicrons but not the extent of lipolysis that accompanies the binding. Removal of divalent cations from the system with EDTA results in a rise both in chylomicron binding and lipolysis. It is suggested that the binding sites are accessible to the lipase that is responsible for hydrolysis.

KEY WORDS rat · liver cells · chylomicrons · uptake · temperature · pH · role of phospholipids · divalent ions

PREVIOUS REPORTS from this laboratory have shown that isolated liver cells will bind chylomicrons and hydrolyze the triglyceride while the chylomicrons are bound to the cell membrane and are effectively outside the cell (1). No further metabolism of the fatty acid occurs. Work in vivo has confirmed these observations and has indicated that the binding studied in vitro is comparable with that occurring in the intact animal (2). Isolated liver cells therefore present a useful tissue preparation for the study of the mechanism of chylomicron binding by liver. The liver cells may be prepared in a homogeneous suspension and may be subjected to enzymatic and chemical modifications that are not possible with the intact organ; yet liver cells do not have the disadvantages of homogenates or tissue slices, which contain cut or broken cells. Further studies of the nature of the uptake of chylomicrons by isolated liver cells are reported here.

METHODS

Isolated liver cells were prepared as described previously (1). They were examined with the light microscope under the different conditions used. Any changes noted are described in the text.

Chylomicrons were obtained from rat thoracic duct lymph as described previously (3). Labeled chylomicrons were prepared in the same way after the animal had been fed 5 μc of glycerol tripalmitate-1-^{14}C in olive oil. Lipid fractionation and counting procedures were as described previously (1, 2).

Standard Incubation Conditions

In all experiments, chylomicrons were incubated with liver cells in a Dubnoff metabolic shaker in Hanks' solution (4) at pH 7.4 unless otherwise stated. Aliquots were taken at a series of times and the cells in them were centrifuged down. The cells were washed by resuspension and recentrifugation and the lipid was extracted (5). Chylomicrons labeled with ^{14}C as above were used unless otherwise stated. All experiments were performed on at least two cell preparations and in duplicate on each cell preparation. Single experiments are represented in the Figures, but these are typical of each group of experiments.

Sialic Acid Determinations

10 ml of isolated liver cells (8 mg of N) in Hanks' solution, pH 7.4, was incubated with an equal volume of 0.8 N H_2SO_4 for 30 min at 80°C. Aliquots were taken before

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and after incubation, the cells were centrifuged down, and the sialic acids in the supernatant fraction were determined by the thiobarbituric acid method of Aminoff (6). A blank determination on cell supernate alone was also performed. A level of 18 μg of sialic acid per mg of cell nitrogen was found, or approximately 1.5 μg/mg dry weight. This is similar to the value reported by Naaman, Eisenberg, and Doljanski (7) of 1.1 μg/mg dry weight.

Liver cells (12 mg of N) were also incubated with neuraminidase (500 IU, Koch-Light Laboratories, Ltd, Colnbrook, Bucks, England) in Hanks' solution (10 ml) for 60 min at 37°C. Aliquots were taken before and after each incubation and the cells were removed by centrifugation. The free sialic acid in each supernatant fraction was determined. This procedure released 27% of the total sialic acids.

Treatment of Chylomicrons

Chylomicrons (13.7 mg of lipid) were treated with phospholipase D (3.25 EU, British Drug Houses Ltd.) as described by Ashworth and Green (8). The chylomicrons were isolated from the medium by centrifugation at 60,000 *g* for 15 min and washed four times by resuspension in 0.14 M NaCl and recentrifugation. Aliquots of the first infranate were taken and the choline was determined by the enneaiodide method (9). Under these conditions 200 μg of choline was released. This represented 89% of the available choline calculated from the amount of phospholipid [determined by the method of King (10)] on the assumption that this is 79% lecithin (11).

Chylomicrons were separated into "membrane" and "fat droplet" fractions as described by Zilversmit (12).

Preparation of Micelles

Micelles of pure lecithin and of lecithin, cholesterol, and triglyceride in the ratio found for the chylomicron "membrane" fraction (i.e. lecithin–cholesterol–triglyceride 7:2:1) were prepared by subjecting the lipids in Hanks' solution, at a concentration of 7 mg/ml, to ultrasonication (13).

RESULTS

Effect of Temperature on the Binding of Chylomicrons

The binding of chylomicrons by liver cells at different temperatures, shown in Fig. 1, is greatest at temperatures below 37°C. The binding was particularly marked at 7°C, although on continued incubation bound lipid was lost from the cells. As the binding of chylomicrons was determined by centrifuging down the cells and resuspending these in Hanks' solution followed by recentrifugation, weakly bound chylomicrons would be removed. The reason for the greater initial binding at 7°C is not clear.

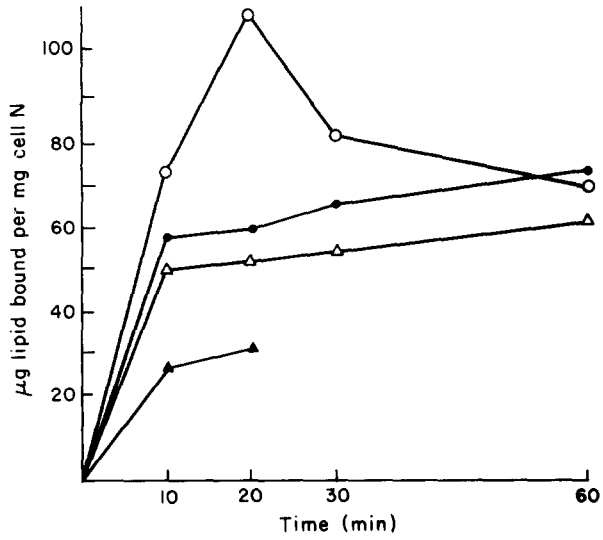

FIG. 1. Effect of temperature on the binding of chylomicrons by liver cells. Isolated liver cells (3.4 mg of N) were incubated with chylomicrons (1.8 mg of lipid) in Hanks' solution (pH 7.4, 5.5 ml) at different temperatures. At 51 °C the cells began to clump after 30 min incubation and a homogeneous aliquot could not be obtained. ○, 7 °C; ●, 24 °C; △, 37 °C; ▲, 51 °C.

Effect of Potential Bond-Breakers on Removal of Bound Chylomicrons from Liver Cells

It has been shown previously that chylomicrons are bound rapidly and firmly to liver cells and are not removed to any significant extent by centrifugal forces in excess of those required to float them when free (1). The effect of centrifuging liver cells, which have bound chylomicrons, in the presence of a number of reagents known to affect the formation and stability of different binding forces was therefore investigated. This method was adopted because it allowed the initial uptake of chylomicrons under physiological conditions and eliminated the possibility of unphysiological binding which might occur in the presence of these reagents.

The reagents used were selected to give a range of effects. Urea and guanidine hydrochloride are both hydrogen-bond breakers (14, 15) and there is evidence that they also modify water structure and consequently hydrophobic bond formation (16, 17). Dioxane and dimethylsulfoxide disrupt hydrophobic bond formation. In addition, dimethylsulfoxide is a strong hydrogen-bonding agent, while dioxane is a weak one and may be considered to act on hydrophobic bonds alone. 1 M sodium chloride was used to test the effect of high ionic strength on binding and hence whether electrostatic or ionic interactions are involved.

On centrifugation of liver cells, having bound chylomicrons, at 20,000 *g* for 60 min, chylomicrons floating to the top of the centrifuge tube represented 10.0% ± 3.6% (SD, n = 6) of the total initially bound. This rose to 42.5% ± 9.2% (SD, n = 4) in the presence of 2 M urea,

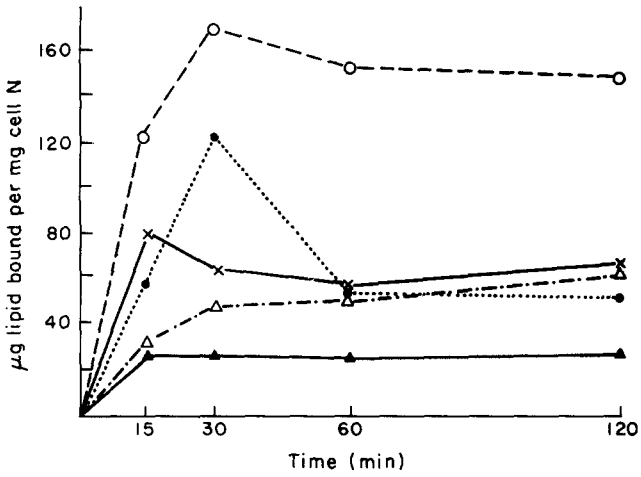

FIG. 2. Effect of pH on chylomicron binding by liver cells. Isolated liver cells (15.1 mg of N) were incubated with chylomicrons (2.75 mg of lipid) at 37 °C in buffered saline at different pH values. ●, pH 2.83; ○, pH 4.32; ×, pH 7.1; △, pH 10.15; ▲, pH 11.1.

while dioxane (15 and 30%), dimethylsulfoxide (15 and 30%), 2 M and 4 M guanidine hydrochloride, 1 M sodium chloride, and 4 M urea had no effect. The increased release of chylomicrons in the presence of 2 M urea is difficult to explain; as it did not occur at the higher urea concentration, the effect is probably a specific one and not due to general disruption of hydrogen or hydrophobic bonds.

Effect of pH on Chylomicron Binding

Liver cells were incubated with chylomicrons in 0.14 M NaCl that contained $CaCl_2$ (0.1 g/liter), KCl (0.4 g/liter) and $MgSO_4.7H_2O$ (0.1 g/liter) buffered by addition of one part in ten of universal buffer (18). At all pH values the cells retained their integrity. However, at pH 11.15 and 2.83 a small amount of clumping occurred, and at the lower of these pH values the cells assumed a more granular appearance. The binding of chylomicrons by liver cells at different pH values is given in Fig. 2.

Binding is greatest at pH 4.32, the isoelectric point of the chylomicrons (19). At this pH the chylomicrons have no net charge and clumping may occur. The increased binding may be due to uptake of groups of chylomicrons or to decreased repulsion between the cells and chylomicrons, both of which are negatively charged at physiological pH's. Both these effects may be operative, and it is not possible to distinguish between them in this experiment. Microscopic examination of the chylomicrons at pH 4.32 did not show clumping to any great extent. However, as binding is only doubled, clumps of two chylomicrons would give this result.

At pH 2.83, when chylomicrons bear a net positive charge, binding still occurs. Uptake shows a peak at 30 min, after which bound lipid is lost from the cells. Such a loss at a physiological pH is produced by hydrolysis of chylomicron triglyceride and transfer of liberated fatty acids into the medium (1). At pH 2.83, when hydrolysis is low, the loss is nevertheless greater than at pH 7.1, and probably represents loss of bound chylomicrons. Possibly binding of positively charged chylomicrons occurs at sites, not normally operative, from which they are lost on continued incubation. A similar effect was found at low temperatures.

These experiments were repeated with a range of different buffers (glycine–NaOH, phosphate, and glycine–HCl). Essentially the same results were obtained as with universal buffer in a similar pH range.

Binding of Chylomicrons Treated with Phospholipase D

The stability of chylomicrons depends on the phospholipid (20), which may form a layer around the central triglyceride droplet (21). This layer would be expected to form the contact with the liver cell surface. Treatment of chylomicrons with phospholipase D reduced binding by liver cells at pH 7.4 by a factor of approximately three at all incubation times tested (up to 2 hr).

Binding of Chylomicrons in the Presence of Chylomicron Fractions

The binding of chylomicrons by liver cells in the presence of chylomicron "membrane" and "fat droplet" fractions (12) is shown in Fig. 3. Binding is reduced in the presence of either fraction. However, in the case of the "fat droplet," the final (2-hr) level is similar to that of the control, while that in the presence of the membrane fraction remains low.

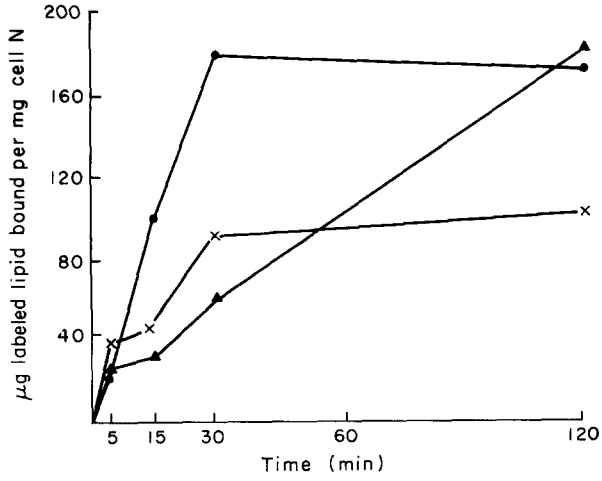

FIG. 3. Binding of chylomicrons by isolated liver cells in the presence of "membrane" and "fat droplet" fractions of chylomicrons. Liver cells (2.5 mg of N) were incubated with labeled chylomicrons (1.9 mg of lipid) at 37 °C in Hanks' solution in the presence of fractions prepared from unlabeled chylomicrons (6 mg). ●, control; ▲, "fat droplet" fraction present; ×, "membrane" fraction present.

Both chylomicron fractions are bound by liver cells. When fractions prepared from 1.3 mg of ^{14}C-labeled chylomicrons were used, binding of the "fat droplet" fraction by liver cells (11 mg of N) occurred to the level of 51 μg/mg N or 52% of the total lipid present, while the "membrane" fraction was bound to the extent of 91%. It was not possible in the latter case to determine the weight of membrane bound, as this was a very small proportion of the original chylomicrons. The results shown in Fig. 3 are probably due to competition between the chylomicrons and each added chylomicron fraction. In the case of the "fat droplets" the chylomicrons are bound more slowly but the final level of uptake is the same as in the case of the control, while in that of the "membrane" the final level of chylomicron uptake is reduced, which suggests that this part of the chylomicron is in contact with the cell during binding. It is difficult to prepare chylomicron fractions in quantity, and complete inhibition of chylomicron uptake by the "membrane" fraction was not achieved. In the experiment shown in Fig. 3, the "membrane" from 6 mg of chylomicrons was incubated in the presence of 1.9 mg of chylomicrons, yet uptake of the latter was reduced only by a factor of two. The intact chylomicron is therefore better adapted than the phospholipids in bulk for the binding at the liver cell surface, perhaps because of a specific orientation of the part of the membrane surface that is involved.

Effect of Phospholipid and Mixed Micelles on Chylomicron Binding

The "membrane" fraction of chylomicrons contains predominantly phospholipid with a small amount of cholesterol, triglyceride, and protein (12). Therefore it was decided to test the possibility that micelles of lipids of the same composition given for the "membrane" fraction and also pure lecithin micelles might inhibit uptake of chylomicrons by liver cells. The results of this investigation are shown in Fig. 4.

Both mixed and pure phospholipid micelles reduce uptake of chylomicrons, the greatest reduction being effected by the lecithin micelles at the highest concentration used. The reduction was not as great as might be expected if the phospholipid micelles had the same structure as the chylomicron "membrane" fraction. However, in the case of micelles the ratio of surface to internal phospholipid would decrease with increased concentration, and the amount of phospholipid that is spatially directed to inhibit uptake of chylomicrons is not represented by concentration alone.

Binding of Chylomicrons by Liver Cells Treated with Neuraminidase

The negative charge in liver cells is due mainly to the presence of sialic acids in the glycoproteins on the cell surface. Treatment of liver cells with neuraminidase caused the level of chylomicron binding to double at all times studied (up to 3 hr). Sialic acids are therefore not involved in binding chylomicrons, but normally reduce their uptake. The result after neuraminidase treatment may simply be due to decreased negative repulsion between the cells and chylomicrons, or it may be more specifically due to removal of glycoproteins, which would allow the chylomicron to come into contact with a greater area of the cell surface.

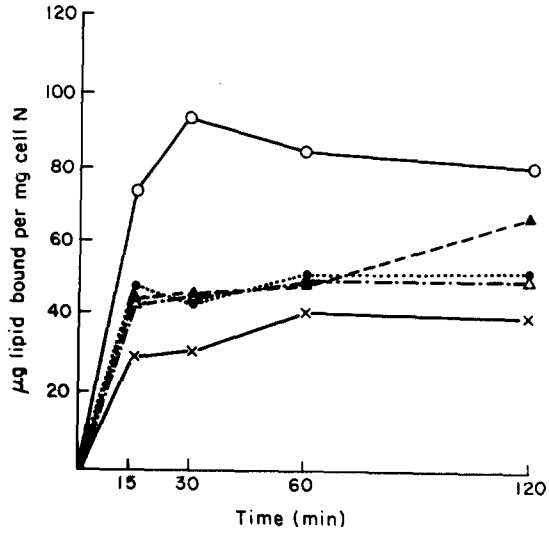

FIG. 4. Effect of micelles on the binding of chylomicrons by liver cells. Isolated liver cells (6.0 mg of N) were incubated with chylomicrons (0.57 mg of lipid) in Hanks' solution (10 ml) at 37 °C, with lecithin or lecithin–triglyceride–cholesterol micelles. ●, lecithin micelles (7 mg); ×, lecithin micelles (45 mg); ▲, lecithin–triglyceride–cholesterol micelles (7 mg); △, lecithin–triglyceride–cholesterol micelles (45 mg); ○, control. Binding of chylomicron lipid only is shown in the figure, although binding of other lipid undoubtedly occurs.

A study of the release of fatty acid from the bound chylomicrons showed that the increased binding is not accompanied by increased hydrolysis. Fatty acid production at 120 min in the control cells was 4.4 μg/mg N and in the treated cells, 3.7 μg/mg N.

Role of Divalent Cations in the Binding of Chylomicrons by Liver Cells

The binding of chylomicrons by liver cells in 0.14 M NaCl, in 0.14 M NaCl containing 5 mM EDTA, or in 0.14 M NaCl containing 5 mM EDTA plus excess (17 mM) $CaCl_2$ is shown in Fig. 5. Removal of divalent cations causes an increased binding of chylomicrons, which indicates that these ions act in some way to prevent uptake. As binding occurs in the presence of 0.14 M NaCl without added calcium, the EDTA must act by removing divalent ions from the cell structure or from the chylomicrons themselves.

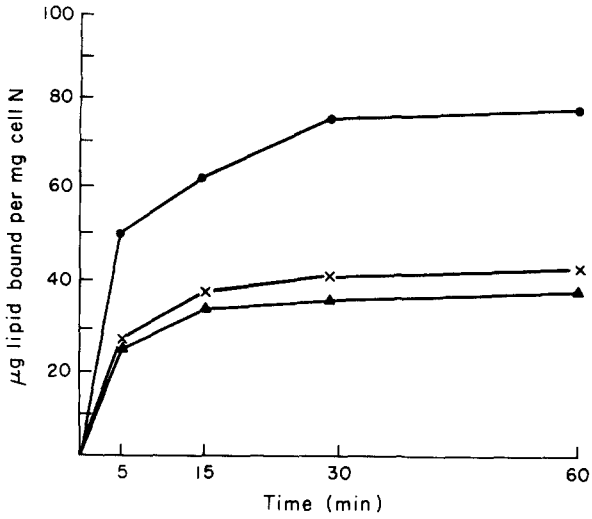

FIG. 5. Role of divalent cations in the binding of chylomicrons by liver cells. Liver cells (12.8 mg of N) were incubated for 15 min at 37°C in 6 ml of 0.14 M NaCl, in 0.14 M NaCl containing 5 mM EDTA, or in 0.14 M NaCl containing 5 mM EDTA and 17 mM $CaCl_2$ (to give 12 mM in excess of EDTA). In addition, chylomicrons (1.23 mg of lipid) were incubated in 5 ml of the above media for the same time. The two suspensions in the same medium were mixed and incubated at 37°C. Preincubation in NaCl, ×; in NaCl + EDTA, ●; and in NaCl + EDCA + $CaCl_2$, ▲.

Production of free fatty acids in the control cells was 3.0 μg/mg N, while in the presence of EDTA this increased to 15 μg/mg N. This increase is proportionally greater than the rise in uptake. Stimulation of lipolytic activity as well as stimulation of binding therefore occurs. It is not possible to tell whether the increased binding is at sites normally operative, or whether the increased lipolysis is only of chylomicrons bound at such sites. In the previous experiment treatment of liver cells with neuraminidase caused an increase in binding but no corresponding rise in lipolysis, which suggests that unphysiological binding was involved. This is not necessarily true in the presence of EDTA.

DISCUSSION

A number of pieces of evidence point to the involvement of phospholipids in the uptake of chylomicrons by liver cells. Binding is reduced by treating the cells with phospholipase D; the membrane fraction of the chylomicrons, which is predominantly phospholipid, inhibits uptake, and both pure and mixed phospholipid micelles also inhibit uptake. Although only the latter point directly implicates the phospholipid alone, these pieces of evidence, taken as a group, lead to the conclusion that chylomicron phospholipids play a role in the binding of chylomicrons by liver cells. In the physiological state chylomicrons have a layer of adsorbed protein. However, this can be largely removed by washing, and the protein that remains is insufficient to form a layer around the chylomicron surface (20, 22). It is therefore likely that, in vivo, at least part of the phospholipid is exposed, and can come into contact with the liver cell surface.

Several experiments were performed in an attempt to elucidate the nature of the forces involved in the binding of chylomicrons by liver cells. The results of these, while not conclusive, yielded a number of useful observations. (*a*) The binding was reduced at higher temperatures, which suggests that thermal agitation weakens the binding forces. (*b*) Since chylomicrons were bound to liver cells in the pH range 2.8–11.1 with a maximum binding at pH 4.32, the isoelectric point of the chylomicrons, the charge of the particles does not seem to be the primary factor determining their attachment to cells. (*c*) Reagents known to decrease the stability of hydrogen bonds, hydrophobic bonds, and ionic bonds had no effect on the release of chylomicrons from liver cells with the exception of 2 M urea, which enhanced their release; 4 M urea had no effect. In view of the complex nature of liver cells and chylomicrons, a precise interpretation of these observations is difficult, but they suggest that Van der Waals forces, which occur readily between approaching bodies (23, 24), might be primarily responsible for the attachment of the particles to liver cells.

Previous results have shown that liver cells bind chylomicrons rapidly and firmly and that cells are saturated when only a fraction of the cell surface is covered (1, 2). In addition, binding of chylomicrons is accompanied by hydrolysis of the triglyceride, catalyzed by an enzyme present in the cell plasma membrane (2). An increased uptake of chylomicrons by cells treated with neuraminidase does not result in hydrolysis of the extra triglyceride bound. It is possible that the lipase is inhibited by neuraminidase. However, if this is true the inhibition is selective and results in a residual lipolytic activity equal to that of the untreated cells. It seems more likely, therefore, that the new sites "uncovered" by treatment of the cells with neuraminidase are not available to the lipase.

At pH 4.3 and after removal of divalent ions, both of which cause increased binding of chylomicrons, increased hydrolysis does occur. However, pH 4.3 is the optimum pH of the lipase (Higgins, J. A., and C. Green, manuscript in preparation) and removal of divalent ions stimulates the enzyme's action, so that it is not possible to tell if this additional hydrolysis is of the extra triglyceride bound at this pH or represents enhanced hydrolysis of the triglycerides bound at the usual sites.

Although these results do not prove that all chylomicrons bound at sites operative under physiological conditions are available for hydrolysis, they do suggest that only chylomicrons bound at these sites are hydrolyzed. It is possible that chylomicrons are transferred from the binding site to a second site where hydrolysis occurs, although as both the binding site and the lipase appear to

be on the cell surface (2) it is not necessary to postulate such a transfer to explain the observed results.

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