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Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages

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In vivo uptake and processing by liver macrophages (Kupffer cells) of liposomes, covalently coated with rabbit immunoglobulin (Ig liposomes) was studied following intravenous injection in rats. Rabbit Ig liposomes were labeled with trace amounts of cholesteryl[^{14}C]oleate and [^{3}H]cholesteryl hexadecyl ether. 1 h after injection of the liposomes, the non-parenchymal cells were isolated and subjected to centrifugal elutriation with stepwise-increasing flow rates; thus, five sub-fractions of Kupffer cells were obtained ranging in size from 9 to 14 μm in diameter. The cells were assayed for peroxidase activity and protein content. Rabbit Ig liposomes were taken up preferentially by Kupffer cells with diameters larger than 11 μm, which constitute less than 25% of the total Kupffer cell population. The intralysosomal degradation of the ingested liposomes was monitored by measuring the $^{3}H/^{14}C$ ratio of the cells. Due to the rapid release from the cells of the [^{14}C]oleate formed from the cholesteryl[^{14}C]oleate and the virtually complete retention of the non-metabolizable [^{3}H]cholesteryl hexadecyl ether the $^{3}H/^{14}C$ ratio of the cells increases with proceeding hydrolysis of the liposomes. Thus, we were able to show that, in vivo, the Kupffer cells of the larger size classes, are not only more active in liposome uptake, but are also substantially more active in liposome degradation than smaller cells. The maintenance of the observed heterogeneity of rat liver Kupffer cells, with respect to liposome uptake under in vitro culture conditions, was examined. Subfractions were maintained in monolayer culture for 2 days and incubated with rabbit Ig liposomes. Binding and uptake of liposomes by the cells was monitored by measuring cell-associated radioactivity at 4°C and 37°C, respectively. In contrast to our in vivo results, we observed maximal in vitro liposome binding and uptake in those subfractions containing small cells (10–11 μm diameter), while the fractions containing cells larger than 12 μm, which were more active in vivo, were substantially less active than the smaller cells. The maximum we observed was even more pronounced when the liposome concentration was increased. We conclude that liver macrophage subfractions that barely participate in liposome uptake from the bloodstream in vivo, possess the potential to develop the capacity in vitro to phagocytose rabbit Ig-coated liposomes to extents equal to or even higher than the cells belonging to those subfractions containing the phagocytically most active cells under in vivo conditions.

Introduction

Hepatic uptake of large unilamellar or multilamellar liposomes is accomplished almost exclusively through phagocytosis by liver macrophages (Kupffer cells) [1]. During in vitro studies with isolated Kupffer cells, we found that modification of the liposomal surface by covalent attachment of

Abbreviations: Ig, immunoglobulin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MPB-PE, maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine; PBS, phosphate-buffered saline.

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rabbit immunoglobulin (rabbit Ig liposomes) allows interaction of the liposomes with the Kupffer cell Fc receptor, and thereby increases the uptake of liposomes by Kupffer cells by a factor of 5–10 [2]. Since Fc receptors also occur on the surfaces of numerous other cell types, it was of interest to study also the fate of the rabbit Ig liposomes in vivo. In addition, in our in vitro studies we obtained morphological evidence of a heterogeneity in Fc receptor density on the Kupffer cells in monolayer culture (Derksen et al. unpublished data). Previous studies by others indicating that the Kupffer cell population in vivo is also functionally heterogeneous [3,4], provided a further incentive to study the in vivo fate of rabbit Ig liposomes and to extend our experiments to the heterogeneity of Kupffer cells with respect to liposome uptake and processing.

Thus, we injected large unilamellar liposomes intravenously into rats, measured uptake by Kupffer cells in relation to cell size and compared this with the uptake of liposomes covalently coated with rabbit immunoglobulin. We sought to confirm the involvement of Fc receptors in the uptake of these liposomes by inhibition experiments, using heat-aggregated human immunoglobulin. In order to enable studies of both uptake and processing of liposomes simultaneously, we used the radioactive label cholesteryl[^{14}C]oleate and its non-metabolizable analog [^{3}H]cholesteryl hexadecyl ether. Since the latter is not released from the cells after it has been taken up in association with liposomes, it is a highly reliable measure of the total amount of liposomes taken up [5,6]. In contrast, the cholesteryl[^{14}C]oleate label is rapidly hydrolyzed upon intracellular uptake and, since the [^{14}C]oleate formed is readily released from the cells [7], the cellular $^{3}H/^{14}C$ ratio provides a convenient and reliable parameter of the extent of liposome processing by the cells.

For comparison, the capacity of subpopulations of Kupffer cells with respect to liposome uptake and processing was also determined under in vitro conditions in monolayer cultures.

Material and Methods

Materials. Egg-yolk phosphatidylcholine (type V-E), cholesterol (type CH-S), dicetylphosphate, bovine-, rabbit-, and human γ-globulin (Cohn fraction II) and Hepes were from Sigma. Cholesteryl[^{14}C]oleate (58 mCi/mmol) was purchased from the Amersham International, and [^{3}H]cholesteryl hexadecyl ether (51.3 Ci/mmol) was from New England Nuclear.

Liposomes. Large unilamellar vesicles were prepared in 10 mM Hepes/135 mM NaCl buffer (pH 7.4) according to Szoka and Papahadjopoulos [8] and sized by extrusion through 200-nm polycarbonate membranes (Nuclepore). The liposomes were composed of egg phosphatidylcholine, cholesterol, dicetylphosphate and maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine (MPB-PE) (synthesized as described by Martin and Papahadjopoulos [9] in a molar ratio of 19:16:4:1. Trace amounts of [^{3}H]cholesteryl hexadecyl ether and cholesteryl[^{14}C]oleate were added to the lipid mixture when required. Rabbit Ig was covalently attached to these liposomes as described previously [10]. Briefly, MPB-PE-containing liposomes (20 μmol total lipid) in 1.0 ml Hepes-NaCl buffer were incubated with 1.0 ml of a solution of 7 mg/ml rabbit Ig, thiolated to 3–4 mol sulfhydryl groups per mol Ig with the heterobifunctional reagent *N*-succinimidyl-*S*-acetylthioacetate [11]. After isolation of the rabbit Ig-coupled liposomes with a flotation method on a dextran gradient [12], the resulting liposomes were assayed for phosphorus and protein content. Rabbit liposomes thus prepared were found to contain between 180 and 200 g rabbit Ig/mol of liposomal lipid. After resuspending the liposomes in Hepes-NaCl buffer, aggregates that might have been formed during the coupling and isolation procedure were removed by forced filtration of the liposomes through a polycarbonate membrane, pore size 0.4 μm. Control liposomes were prepared from the same lipid mixture but, instead of with rabbit Ig, they were incubated with cysteine in a molar concentration twice that of MPB-PE, to block reactive maleimide groups on the liposome surface.

Animal experiments. Elimination of control- and rabbit Ig liposomes from blood was determined in unanesthetized, unrestrained Wistar rats (250–300 g) equipped with permanent heart catheters [13]. Liposomes were injected intracardially by means of the heart catheter at a dose of 1 μmol liposomal

lipid/100 g body weight. In some experiments, aggregated human Ig (10 mg in 0.5 ml Hepes-NaCl buffer) was injected 5 min prior to the liposomes.

To study the liposome distribution in Kupffer cell subpopulations, liposomes were injected into the tail vein of female Wistar rats (170–210 g) under mild ether anesthesia at doses of 1 μmol liposomal lipid/100 g body weight, in some experiments after a preinjection of aggregated human Ig (10 mg/animal), 5 min prior to the liposomes. 1 hour after the injection of liposomes livers were perfused in situ with Gey's balanced salt solution and Kupffer cells were isolated.

Kupffer cells. Kupffer cells were isolated from female Wistar rats (170–210 g) by pronase digestion of the liver and purified by centrifugal elutriation, basically according to Knook and Sleyster [14] except that after the isolation of non-parenchymal cells by metrizamide gradient centrifugation, Kupffer cells were separated in a range of size classes by increasing the buffer flow rate in the elutriation system stepwise at a constant rotor speed (2500 rpm) after being flushed in at 18.0 ml/min. The flow rates used were 20.5, 25.0, 30.0, 35.0, 40.0 and 46.5 ml/min. These fractions were designated 0, A, B, C, D and E, respectively. At each flow rate, a cell suspension of 100 ml was collected. Cells were pelleted and resuspended in phosphate-buffered saline. One sample was used for protein determination according to Lowry et al. [15]. Bovine globulin was used as a standard. Another sample was diluted in Isoton® (Coulter electronics) and cells were counted with a Coulter counter (Coulter electronics) at threshold, sensitivity and aperture settings of 5, 2 and 64, respectively. ^{3}H and ^{14}C radioactivity in a third sample was counted in Plasmasol (Packard) with an LKB scintillation counter.

For in vitro experiments $6 \cdot 10^5$ cells of each subfraction were plated out in 24-well culture plates and cultured in DMEM, containing 20% fetal calf serum as described previously [2]. To obtain adequate amounts of cells for plating, elutriation subfractions were pooled in some experiments. After 2 days in culture, cells were used in liposome uptake experiments.

Incubations. Unless indicated otherwise, in vitro incubations were performed in quadruplicate in 0.5 ml medium without antibiotics or serum. Aggregated human immunoglobulin was added to the incubation medium 10 min before addition of the liposomes at a final concentration of 1 mg/ml. Liposomes were added to the medium as concentrated suspensions. At the end of the incubation time, the incubation medium was pipetted off and the cells were washed six times with cold phosphate-buffered saline (pH 7.4). Cells were digested in 0.7 ml NaOH (0.5 M) and assayed for protein and radioactivity.

Other methods. Aggregated human immunoglobulin was prepared by heating a solution of human immunoglobulin (20 mg/ml in 10 mM Hepes/135 mM NaCl buffer (pH 7.4)) for 30 min at 63° C. Subsequent centrifugation for 15 min at $10000 \times g$ was employed to remove large aggregates. The aggregated human Ig suspension was then sterilized by filtration through sterile membrane filters of 0.22 μm pore size (Millipore). Phospholipid phosphorus was determined after perchloric acid destruction according to Böttcher [16].

Results

From Fig. 1a it is evident that rabbit Ig liposomes are cleared from the bloodstream much faster than control liposomes. Pre-injection of 10 mg aggregated human immunoglobulin does not appear to inhibit the clearance of rabbit Ig liposomes to a significant extent.

After injection of the control liposomes the $^{3}H/^{14}C$ ratio in the blood remains approx. 1.0 throughout the 4 h monitoring period. In contrast, the ratio of the label in rabbit Ig liposomes in blood remains constant for only 30 min after injection and from then on declines (Fig. 1b). This pattern remains unchanged when the animals receive a pre-injection of aggregated human Ig, except for a small, unexplained increase immediately after injection of the rabbit Ig liposomes.

1 h after injection, when nearly all rabbit Ig liposomes have been cleared from the blood, the liver has accumulated 80–85% of the injected label, irrespective of prior injection of aggregated human Ig (Table I). At that time, control liposomes have not yet been eliminated from the blood to the same extent and concomitantly, in that case hepatic uptake is only about 65% of the injected

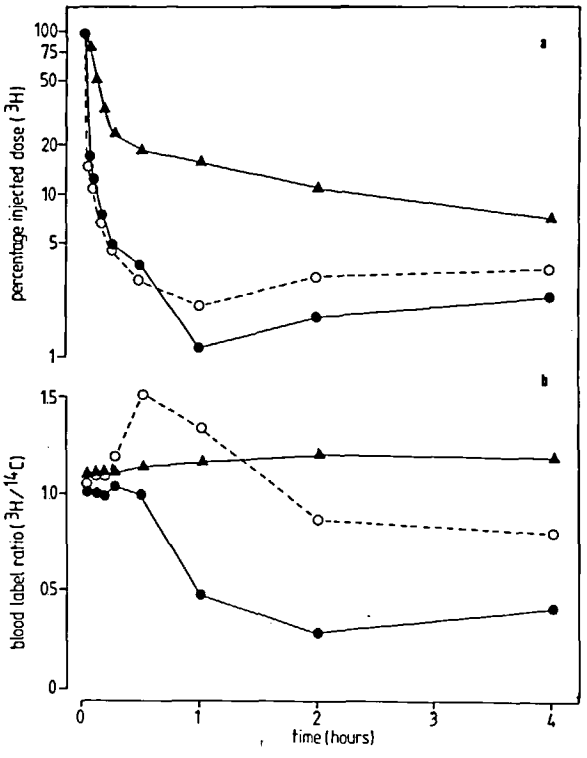

Fig. 1. Disappearance of the ^{3}H label (A) and the ^{3}H/^{14}C ratio (B) was monitored in blood samples from rats equipped with a permanent heart catheter after injection of [^{3}H]cholesteryl hexadecyl ether/cholesteryl[^{14}C]oleate labeled control (▲) or rabbit Ig-liposomes (●, ○), with (○) or without (●) prior injection of 10 mg aggregated human Ig. The initial liposome label ratio was 1.0.

dose, again irrespective of prior injection of aggregated human Ig. The relative contribution of the spleen in liposome uptake is notable different in the two types of liposome; the ratio of splenic and hepatic uptake is 2-fold higher in control liposomes than in rabbit Ig liposomes. As regards the intracellular processing of the liposomes, the ^{3}H/^{14}C ratios shown in Table I seem to indicate that liver and spleen degrade both types of liposome at the same rate, the ratios being similar for the control and rabbit Ig-liposomes. A small difference between liver and spleen appears to arise upon pre-injection of aggregated human Ig, which for the liver causes a decrease and for the spleen an increase in the isotope ratio and thus, in metabolic activity. The isotopic ratio in liver and spleen increases approx. 2-fold compared to the initial ratio in the liposomes. This means that after 1 h, half of the ^{14}C label that has entered the cells in association with the liposomes has been shed from the cells into the bloodstream and redistributed to other organs.

In a number of studies we have shown that the principal liver cell type involved in the uptake of liposomes from the bloodstream is the Kupffer cell. Isolation of a total Kupffer cell population by elutriation centrifugation involves the collection of all cells flushed out of the rotor chamber at a flow rate of 46.5 ml/min after the endothelial cells have been flushed out at 20.5 ml/min. In the

TABLE I

LABEL RECOVERY 1 H AFTER INJECTION OF LIPOSOMES

Rabbit Ig (RbIg) or control liposomes, doubly labeled with [^{3}H]cholesteryl hexadecyl ether and cholesteryl[^{14}C]oleate were injected into the tail veins of female Wistar rats, some of which had received an injection of 10 mg aggregated human immunoglobulin (aggHumIg) 5 min prior to liposome injection. 1 hour after injection, the rats were killed and radioactivity was determined in blood, total liver and spleen. Results are means ± S.D. of four rats.

	Blood		Total liver		Spleen	
	% ^{3}H recovery	^{3}H/^{14}C ratio	% ^{3}H recovery	^{3}H/^{14}C ratio	% ^{3}H recovery	^{3}H/^{14}C ratio
RbIg-liposomes	0.7±0.1	0.5±0.2	85.1±6.7	1.7±0.2	4.8±0.7	1.7±0.1
RbIg-liposomes + aggHumIg	1.9±0.2	1.3±0.2	82.3±8.8	1.5±0.1	3.9±0.8	2.4±0.2
Control liposomes	16.2±3.8	1.1±0.1	62.2±4.2	1.9±0.1	8.6±1.4	1.6±0.4
Control liposomes + aggHumIg	6.1±2.7	0.8±0.1	67.2±8.9	1.3±0.1	5.8±1.2	2.1±0.4

present study, however, we increased the counter flow rate stepwise between 25.0 ml/min and 46.5 ml/min which allowed us to isolate five sub-fractions of Kupffer cells ranging in diameter from about 9 to about 14 μm (Table II). Starting the elutriation of a crude non-parenchymal cell fraction at a flow rate of 18.0 ml/min, mostly small cells such as lymphocytes, cellular debris and blebs are collected in this fraction. The next fraction, at 20.5 ml/min, fraction 0, consists of cells of which the majority is peroxidase negative and also fails to attach to culture plates. This fraction is therefore likely to consist mostly of endothelial cells, as has also been reported previously [14]. The cells in fractions A–E (Table II) are nearly all peroxidase positive, albeit with different intensities, and readily attach to culture plates. Consequently, these cells are considered to be Kupffer cells of different sizes. The relatively small cells in fraction A, although able to attach to culture dishes, stain only very faintly when assayed for peroxidase activity, in contrast to the larger cells in fractions D and E which stain heavily. The cells in the fractions in between, showed a gradually increasing stain intensity; thus, there appears to be a correlation between cell size and specific peroxidase activity. Although peroxidase activity is widely used as a parameter for the identification of Kupffer cells, we feel that this may become questionable for cells isolated in fraction A. Staining is so faint in these cells, especially upon comparison with fractions D and E, that the designation as a Kupffer cell may become arbitrary without the use of an additional parameter. The ability, after 1 day in culture, to phagocytose particles such as liposomes may be considered to serve this purpose. Based upon these criteria, i.e., (faint) peroxidase staining, attachment to culture dishes and subsequent phagocytic potential, virtually all cells isolated in fraction A–E can be considered macrophages. The recovery of cells was different in the various fractions, as indicated in Table II. The majority of cells was recovered in fractions A and B; fractions C–E containing progressively fewer cells.

Upon injection of labeled liposomes, the label distribution in the various fractions showed a maximum in the fractions B–D for control liposomes and in C and D for the rabbit Ig liposomes (Fig. 2a). The differences in uptake, however, between the control and protein-coated liposomes were not statistically significant in any of the subfractions, with or without prior injection of aggregated human Ig. If liposome uptake is calculated on the basis of cell number, we obtain results, as presented in Fig. 2b, showing a more consistent correlation between the diameter of the cell and the extent of liposome uptake, i.e., uptake increases progressively with cell size. The highest liposome uptake per cell was found for rabbit liposomes without prior injection of aggregated human Ig. The uptake per cell of both types of

TABLE II

KUPFFER CELL SUBPOPULATIONS AFTER FRACTIONATED ELUTRIATION

Kupffer cells were obtained by pronase digestion of hepatocytes followed by centrifugal elutriation of the resulting non-parenchymal cell fraction with stepwise increasing flow rate. This resulted in Kupffer cell populations of increasing diameter; n.d., not determined.

Fraction number	Elutriation flow rate (ml/min)	Cell size class * (diameter, μm)	Recovery (%) (mean ± S.D.; $n = 16$)	Peroxidase positive cells (%)	Cellular protein (pg/cell) (mean ± S.D.; $n = 16$)
0	20.5	8.7–9.2	n.d.	35	n.d.
A	25.0	9.2–10.2	49.4 ± 3.4	85	53.4 ± 5.4
B	30.0	10.2–11.2	26.4 ± 2.8	86	75.7 ± 8.0
C	35.0	11.2–12.0	11.0 ± 1.6	88	97.6 ± 10.0
D	40.0	12.0–13.0	8.3 ± 1.4	95	120.1 ± 11.0
E	46.5	13.0–14.0	5.0 ± 1.4	95	141.9 ± 11.0

* Calculated from the nomogram supplied by the manufacturer of the elutriation centrifuge (Beckman Inc.).

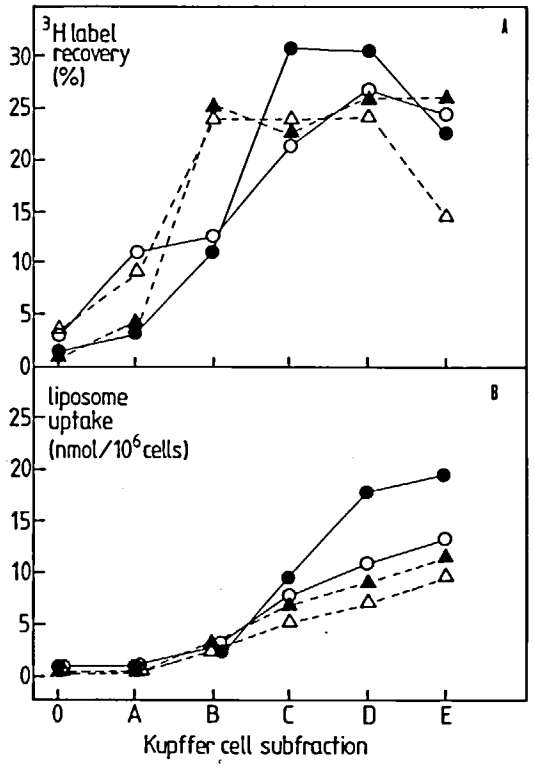

Fig. 2. Uptake of liposomes by Kupffer cells of different size classes as calculated from cell associated [^{3}H]cholesteryl hexadecyl ether. Rabbit Ig (●, ○) or control liposomes (▲, △) were injected intravenously with (○, △) or without (●, ▲) a prior injection of 10 mg aggregated human Ig. 1 h after injection, Kupffer cells were isolated, counted and assayed for radioactivity.

liposome was somewhat reduced as a result of pre-injection of aggregated human Ig, particularly in the larger cells.

We also compared the digestive capacity of the subfractions of Kupffer cells towards the internalized liposomes. The increase in the $^3H/^{14}C$ ratio in the cells provides a good measure of the extent to which the liposomes taken up by the cells were degraded, as outlined in the Introduction. Fig. 3 shows that there is a substantial difference between the subfractions with respect to metabolic activity, particularly towards the control liposomes, for which we observed a $^3H/^{14}C$ ratio 3-fold higher in fraction B than in fraction A cells. For rabbit Ig liposomes, this difference was less marked, while for nearly all subfractions, the extent of degradation of the control vesicles was approx. twice as high as that of the rabbit Ig-coated vesicles. Probably the protein coat has to be degraded by proteolytic enzymes before the liposomal lipids can be hydrolyzed.

Particularly remarkable is the effect of pre-injection of aggregated human Ig on the isotopic ratio. For both the control and the coated liposomes, the increase in the $^3H/^{14}C$ ratio was largely suppressed as a result of injection of 10 mg aggregated human Ig.

Upon culture, almost all cells from the different subfractions became attached to the culture plates. After 2 days in culture, all the attached cells had spread and cell cultures were used in experiments.

As shown in Fig. 4, the amount of cellular protein remaining on the culture dishes after completion of the experiments varied with cell size. It is difficult to determine whether this is a result of protein content difference per cell or a difference in the number of cells remaining attached to the culture dishes. If we compare the amount of protein per cell for the various subfractions with the cellular protein contents immediately after cell isolation (Table II), we see that the ratio in protein content between large (fraction E) and small (fraction A) cells has decreased from 2.6 to 1.3

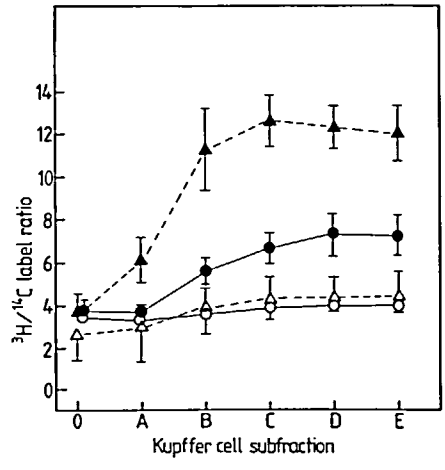

Fig. 3. The $^3H/^{14}C$ ratio was determined in Kupffer cells of different size classes, 1 hour after injection of [^{3}H]cholesteryl hexadecyl ether/cholesteryl[^{14}C]oleate-labeled rabbit Ig (●, ○) or control liposomes (▲, △), with (○, △) or without (●, ▲) prior injection of 10 mg aggregated human Ig. The $^3H/^{14}C$ ratio was normalized for the liposome label ratio. Data presented are means ± S.D. ($n = 4$).

after 2 days in culture. Since the loss of cells due to improper binding to the culture dishes is particularly noticeable in the cells from fraction A, it is likely that the actual difference in protein content per cell is even less than would appear from the results shown in Fig. 4. This implies that during the 2 days in culture, the various Kupffer cell subfractions may behave differently. It is conceivable that especially the smaller cells mature, under these conditions into larger, more differentiated cells, thus levelling out the differences in size and function as observed immediately upon cell isolation.

Fig. 5 shows the uptake of liposomes by Kupffer cell subfractions that have been in culture for 2 days. For all subfractions both uptake at 37° C and binding at 4° C can be inhibited to a substantial extent by pre-incubation of the cells with aggregated human immunoglobulin, as was also seen previously for the whole Kupffer cell population [2].

Upon increasing the liposome concentration in the incubation medium, liposome uptake in all subfractions increased (Fig. 6). This implies that in none of the subfractions, was maximal uptake capacity reached at the concentrations used. Also in this set of experiments we observed a minimum in uptake in fraction B, which originally contained small Kupffer cells. This minimum is more prominent with an increasing liposome concentrations, but only when using rabbit Ig liposomes.

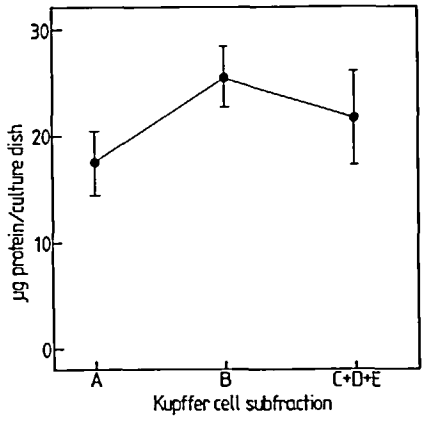

Fig. 4. Protein content of culture dishes. Kupffer cells, isolated in a various size classes and cultured for 2 days, were incubated with liposomes. Unbound liposomes were removed, cells were washed six times with PBS and protein was determined. Data presented are means ± S.D. from 30 individual cultures.

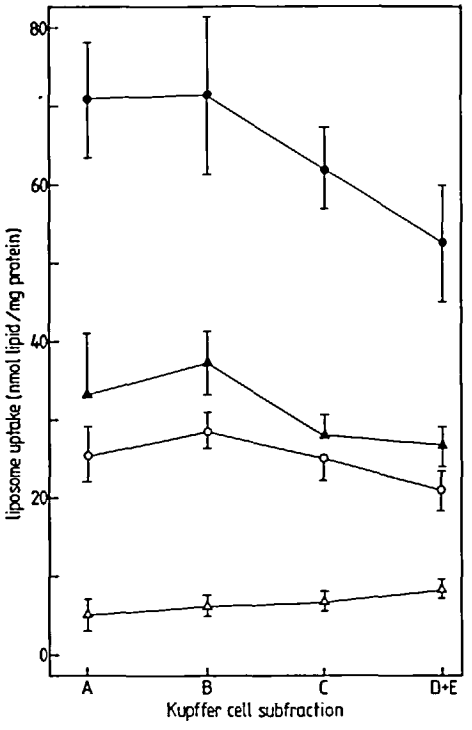

Fig. 5. Rabbit Ig liposome uptake by Kupffer cells isolated into different size classes. After 2 days in culture Kupffer cells were incubated with [^{3}H]cholesteryl hexadecyl ether labeled rabbit Ig liposomes (50 nmol total liposomal lipid/ml) at 37° C (●, ○) or 4° C (▲, △) in the absence (●, ▲) or presence (○, △) of 1 mg/ml aggregated human immunoglobulin. After 4 h of incubation, Kupffer cells were harvested and cell-associated ^{3}H radioactivity was determined. Data represent means ± S.D. of four incubations.

Discussion

The lack of an inhibitory effect of aggregated human Ig on plasma clearance of rabbit Ig liposomes which we observed in this study may have several causes. Firstly, the capacity of Kupffer cells in vivo to phagocytose liposomes may be considerably larger than the capacity in vitro; so much larger, in fact, that any specific interaction with Fc receptors by the immunoglobulin coating is obscured. The increase in total liver uptake observed, which also occurs despite pre-injection of aggregated human Ig, may be due to an increase in liposomal diameter or surface charge by

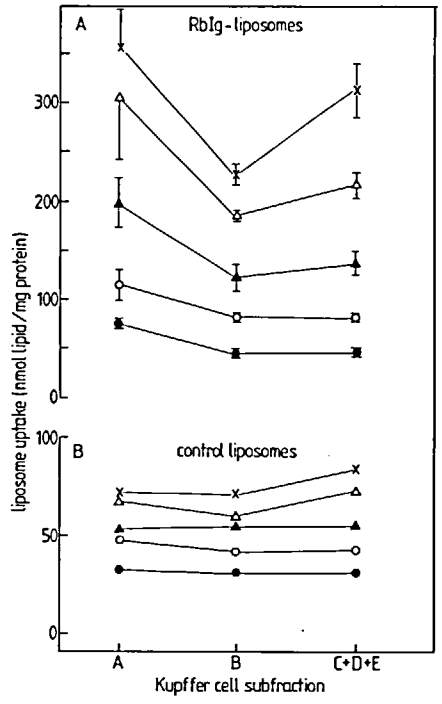

Fig. 6. Concentration dependence of Kupffer cell liposome uptake. Kupffer cells, cultured for 2 days after isolation into different size classes, were incubated for 4 h at 37°C with increasing concentrations of [^{3}H]cholesteryl hexadecyl ether-labeled rabbit (Rb) Ig (panel A) or non-coated liposomes (panel B). The liposome concentrations used were 50 (●), 100 (○), 200 (▲), 400 (△) nd 600 (×) nmol total liposomal lipid/ml. Non-bound liposomes were removed, cells were washed with PBS and cell-associated radioactivity was determined (results are means of four incubations).

the protein coat per se. Secondly, the concentration of monomeric immunoglobulin in the in vivo situation is approx. 20 mg/ml [17], whereas in the experiments in vitro presented here no serum and thus no immunoglobulin was present. Interaction of this monomeric immunoglobulin with Fc receptors is likely to affect the interaction of rabbit Ig liposomes with Kupffer cell Fc receptors in vivo [18]. This is supported by the finding that in humans, the Fc-dependent clearance rate of antibody-sensitized cells was inversely correlated to the serum monomeric IgG concentration [19]. Control experiments, in which we incubated rabbit Ig liposomes in vitro in medium with increasing concentrations of rat serum, also showed an increasing inhibition of Fc receptor-dependent uptake of these liposomes by Kupffer cells (results not shown).

Finally, the amount of aggregated human Ig injected may have been inadequate to block the Fc receptors for a sufficiently long period. This is supported by the finding that aggregated swine immunoglobulins effectively block binding of immune complexes to Fc receptors in vivo only transiently [20].

During the first 20 min after injection, the rate of clearance of the ^{3}H and ^{14}C labels are identical, as judged by the constant ^{3}H/^{14}C ratio in plasma. The subsequent drop in the isotopic ratio, which is most likely caused by release of [^{14}C]oleate from liver and spleen, is quantitatively of little relevance, since it refers to time points at which only very small amounts of liposomes remain in circulation. The label ratio in the total liver sample did not increase substantially, indicating that even though the ^{14}C label is released from the Kupffer cells in the form of ^{14}C-labeled fatty acids (Ref. 7 and Derksen, J.T.P., unpublished results), only a minor fraction of the ^{14}C label leaves the liver during the first hour after injection of the liposomes. This suggests an extensive re-utilization of these fatty acids by the hepatocytes. The latter incorporate [^{14}C]oleate mainly into triacylglycerols [21,22]; thus, the ^{3}H/^{14}C ratio in total liver may eventually rise due to a release of triacylglycerols associated with very-low-density lipoproteins. The drop in label ratio after pre-injection of aggregated human Ig is somewhat smaller than in the control experiment. This may suggest that in this case processing of rabbit Ig liposomes and the subsequent release of the ^{14}C label from the cells is slower (confirmed by the results shown in Fig. 3). The transient increase in isotopic ratio between 20 and 40 min is not likely to be due to a relative loss of the ^{14}C label as a result of cholesterylester exchange, since rats do not possess the exchange protein required for such a process [23]. Perhaps this phenomenon is a reflection of some heterogeneity in the liposome preparation with respect to label distribution. The absence of a drop in the label ratio after injection of the control liposomes is probably related to the less rapid clearance of these liposomes. A small amount of ^{14}C label released into the bloodstream is not likely to have a substantial effect on the ^{3}H/^{14}C label ratio

when there is still a relatively large amount of liposomes present.

Quantitatively, the uptake by the liver of both control and rabbit Ig liposomes can be attributed mainly to a relatively small number of large Kupffer cells. There is, however, a slight tendency towards a shift in uptake of rabbit Ig liposomes to the large Kupffer cells as compared to the control liposomes. Whether this is due to a difference in, for example, Fc receptor density remains to be seen. On a per cell basis, liposome uptake appears to increase progressively with cell size. It can be noticed that the uptake of rabbit Ig liposomes is more dependent on cell size than that of the control liposomes. Also the rate of processing of phagocytosed liposomes shows a dependence on cell size, as reflected by the shift in (cellular) $^{3}H/^{14}C$ ratio. This shift is much larger for control liposomes than for rabbit Ig liposomes which can be explained by assuming that the immunoglobulin coat retards the degradation of the liposomal lipids. The lowering of the $^{3}H/^{14}C$ ratio after previous administration of aggregated human Ig may also be due to a saturation of the Kupffer cell lysosomal system with this protein, causing a delay in the degradation of the liposomes.

It has been shown by Sleyster and Knook that Kupffer cells, localized periportally, are larger and show higher lysosomal enzyme activities than those localized perivenously [3]. They also showed that the periportal Kupffer cells were the ones most actively involved in latex particle phagocytosis. This was independent of the direction of perfusion, which implies an inherent difference in phagocytotic capacity between periportal and perivenous Kupffer cells. In fluorescence microscopic studies using rhodamine phosphatidylethanolamine-labeled liposomes, we also observed preferential accumulation of fluorescence in periportal Kupffer cells (not shown).

The substantial differences in liposome uptake between the different Kupffer cell subfractions are not reflected in our in vitro experiments. If we assume that a similar process underlies the phagocytosis of liposomes and latex particles [24], it is likely that this in vivo difference is an inherent property of the cells and not a result of their sinusoidal location. The lack of difference in the extent of rabbit Ig liposome binding and uptake between the Kupffer cell subfractions, implies that at that time there are no major differences in the expression of Fc receptors at the cell surface by the Kupffer cells of the subfractions used. We tentatively explain this discrepancy by postulating that during the 2-day culture period, the small cells develop towards a more fully mature type of macrophage, thus equalizing the phagocytic capacity of the cells in the different size categories.

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References

1 Roerdink, F., Dijkstra, J., Hartman, G., Bolscher, B. and Scherphof, G. (1981) Biochim. Biophys. Acta 677, 79–89.
2 Derksen, J.T.P., Morselt, H.W.M., Kalicharan, D., Hulstaert, C.E. and Scherphof, G.L. (1987) Exp. Cell Res. 168, 105–115.
3 Sleyster, E.Ch. and Knook, D.L. (1982) Lab. Invest. 47, 484–490.
4 Daemen, T., Veninga, A., Roerdink, F.H. and Scherphof, G.L. (1988) in Liposomes as Drug Carriers: Trends and Progress. (Gregoriadis, G., ed.), pp. 431–445, John Wiley & Sons, New York.
5 Stein, Y., Kleinman, Y., Halperin, G. and Stein, O. (1983) Biochim. Biophys. Acta 750, 300–305.
6 Goodman, DeW., Stein, O., Halperin, G. and Stein, Y. (1983) Biochim. Biophys. Acta 750, 223–230.
7 Derksen, J.T.P., Morselt, H.W.M. and Scherphof, G.L. (1987) Biochim. Biophys. Acta 931, 33–40.
8 Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
9 Martin, F.J. and Papahadjopoulos, D. (1982) J. Biol. Chem. 257, 286–288.
10 Derksen, J.T.P. and Scherphof, G.L. (1985) Biochim. Biophys. Acta 814, 151–155.
11 Duncan, R.J.S., Weston, P.D. and Wrigglesworth, R. (1983) Anal. Biochem. 132, 68–73.
12 Heath, T.D., Macher, B.A. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 640, 66–81.
13 Kuipers, F., Havinga, R., Bosschieter, H., Toorop, G.P., Hindriks, F.R. and Vonk, R.J. (1985) Gastroenterology 88, 403–411.

14 Knook, D.L. and Sleyster, E.Ch. (1976) Exp. Cell Res. 99, 444–449.
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
16 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
17 Lindmark, R., Thoren-Tolling, K. and Sjoquist, J. (1983) J. Immunol. Methods 62, 1–13.
18 Leslie, R.G.Q. (1985) Immunol. Lett. 11, 153–158.
19 Kelton, J.G., Singer, J., Rodger, C., Gauldie, J., Horsewood, P. and Dent, P. (1985) Blood 66, 490–495.
20 Van der Laan-Klamer, S.M., Harms, G., Atmosoerodjo-Briggs, J.E., Hardonk, M.J. and Hoedemaker, Ph.J. (1986) Scand. J. Immunol. 23, 441–447.
21 Nagelkerke, J.F. and Van Berkel, Th.J.C. (1986) Biochim. Biophys. Acta 875, 593–598.
22 Storm, G., Roerdink, F.H., Steerenberg, P.A., De Jong, W.H. and Crommelin, D.J.A. (1987) Cancer Res. 47, 3366–3372.
23 Tall, A.R. (1986) J. Lipid Res. 27, 361–367.
24 Dijkstra, J., Van Galen, M. and Scherphof, G. (1985) Biochim. Biophys. Acta 813, 287–297.