Biochemical and Biophysical Research Communications **256,** 57–62 (1999)
Article ID bbrc.1999.0290, available online at http://www.idealibrary.com on IDEAL®

Uptake of Liposomes Containing Phosphatidylserine by Liver Cells *in Vivo* and by Sinusoidal Liver Cells in Primary Culture: *In Vivo–in Vitro* Differences

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Received January 25, 1999

The interaction with liver cells of liposomes containing different mol fractions of phosphatidylserine was investigated *in vivo* and *in vitro.* Increasing the amount of liposomal phosphatidylserine from 10 to 30 mol% leads to a faster blood disappearance of the liposomes. Within the liver, which is mainly responsible for this elimination, these liposomes are only taken up by the hepatocytes and Kupffer cells. By contrast, sinusoidal endothelial cells, *in vitro,* do bind and internalize liposomes containing ≥30% phosphatidylserine at least as actively as Kupffer cells. The uptake by endothelial and Kupffer cells is inhibited by poly(inosinic acid) and other anionic macromolecules, suggesting the involvement of scavenger receptors. The lack of liposome uptake by endothelial cells under *in vivo* conditions can be attributed to plasma effects since addition of various sera caused severe reduction of *in vitro* uptake of liposomes. *In vivo* the phosphatidylserine head groups may be masked by plasma proteins adsorbed to the liposomal surface, thus preventing recognition by receptors, which are intrinsically able to recognize phosphatidylserine. © 1999 Academic Press

It is widely believed that, *in vivo,* liposomes containing phosphatidylserine (PS) are cleared rapidly from the blood compartment predominantly by cells of the mononuclear phagocyte system (MPS), which in the liver is strongly represented by the Kupffer cells (1). In addition, small liposomes can also be eliminated by the parenchymal cells of the liver, the hepatocytes (2). The mechanisms involved in these clearance activities thus far have remained unclear. Scavenger receptors (ScR) have been implicated in this clearance process, mostly on the basis of *in vitro* studies (3). Certain types of ScR were shown to interact with PS-containing liposomes (3), whereas others, like the so-called classical ScR, were reported not to bind and internalize PS-liposomes (4).

In rat and in human liver, ScR have been identified on sinusoidal endothelial cells, Kupffer cells as well as, to a small extent, hepatocytes (5, 6). Despite the abundant presence of ScR on liver endothelial cells, we have consistently found these cells not to participate in liposome clearance to a significant extent, irrespective of liposomal lipid composition (7). However, recently we found that liposomes surface-modified with anionized albumins are very rapidly removed from the blood by a ScR-mediated process and accumulate predominantly in the endothelial cells (8, 9).

In virtually all of our studies using PS-containing liposomes, as well as in those of most other investigators, PS represented only 10 mol% of the total liposomal lipid. Since surface charge density may be an important determinant in ScR-mediated uptake (10), in the present study we investigated the uptake of liposomes carrying mole fractions of PS from 0 to 100% by the various cell populations in the liver following intravenous administration. The results were compared with those of uptake studies in maintenance cultures of isolated rat liver endothelial cells and Kupffer cells.

MATERIALS AND METHODS

Materials. Phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham AL, USA). Cholesterol (Chol) was from Sigma (St. Louis MO, USA). [^{3}H]cholesteryl oleyl ether was from Amersham (Buckinghamshire, UK). Collagenase (type A), endothelial cell growth factor and collagen S (type 1) were obtained from Boehringer Mannheim (Germany). Fetal bovine serum was purchased from Gibco (Paisley, Scotland, UK) and human serum was obtained from the blood of healthy volunteers. Albumin chemically modified with cis-aconitic anhydride was prepared as described before (6) and was a gift of Prof. Dr. D.K.F. Meijer (Groningen, The Netherlands). All other chemicals were analytical grade or the best grade available.

Methods. For liposome preparation, lipids from stock solutions of PC, PS and Chol in chloroform:methanol (9:1), were mixed in the

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0006-291X/99 $30.00

indicated molar ratio, dried under reduced nitrogen pressure, dissolved in cyclohexane and lyophilized. A trace amount of [^{3}H]cholesteryl oleyl ether was added to each preparation as a non-degradable marker. The liposomes were then hydrated in HN-buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 135 mM NaCl, pH 6.7) and sized by repeated extrusion (13 times) through polycarbonate filters with a pore size of 50 nm (Costar, Cambridge, MA, USA). Phospholipid phosphorus of each liposome preparation was assessed by phosphate assay after perchloric acid destruction (11). Size and size distribution of the liposomes were determined by dynamic laser light scattering with a Nicomp model 370 submicron particle analyzer (NICOMP particle sizing systems, Santa Barbara, CA, USA). The diameter of the liposome preparations used, which was obtained from the volume distribution curves produced by the particle analyzer, was 75.1 ± 3.2 nm (n = 17, mean ± sd). No differences in liposome diameter were observed between liposomes with different PS contents.

For *in vivo* studies, 1 μmol (total lipid) per 100 g of body weight of radio-labeled liposomes was injected into male Wag/Rij rats (200–250 g) via the penile vein under pentobarbital anesthesia. At the indicated times blood samples were taken and 15 or 120 minutes after injection liver and spleen were removed and processed for measurement of radioactivity as described before (8). The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = [0.0219 × body weight (g)] + 2.66 (12). For determination of liposome uptake by the different liver cell types, parenchymal, endothelial cells and Kupffer cells were isolated after collagenase perfusion (13), followed by centrifugation and counterflow centrifugal elutriation as described before (14). The number of cells of the parenchymal, endothelial and Kupffer cell fraction was determined microscopically and the cell-associated radioactivity was determined.

For *in vitro* studies, liver endothelial and Kupffer cells were isolated under semi-sterile conditions after collagenase perfusion as described above. After isolation the cells were washed with RPMI-1640. Kupffer cells were cultured in 24-well cluster plates at a density of 0.75 10^6 cells per well in RPMI-1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin in a humidified 5% CO_2/95% air atmosphere. Liver endothelial cells were cultured in collagen-coated (20 μg/well) 24-well cluster plates in RPMI-1640 supplemented with 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10 ng/ml endothelial cell growth factor in a humidified 5% CO_2/95% air atmosphere (15). The media, now containing 10% FCS, were refreshed after 20 h and every 24 h thereafter. Experiments with the cultured cells were performed on the third day after plating the cells. One hour before the start of the experiment, culture medium was substituted by serum free medium. Experiments were started by removing the medium and adding new medium containing the indicated amounts of [^{3}H]cholesteryl oleyl ether labeled liposomes and indicated additions. Cells were incubated for 3 h at 37°C to determine cell association. After the incubation the culture plates were placed on ice and washed 6 times with ice-cold phosphate-buffered saline, pH 7.4 (PBS). The cells were then lysed in 0.1 M NaOH and cell-associated radioactivity was determined. Protein content was measured according to Lowry (16). When appropriate, statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test.

RESULTS

Blood Elimination

Figure 1 shows the elimination from serum of liposomes with a diameter of 75 nm containing 0%, 10% or 30% PS. Liposomes containing 30% PS are almost completely cleared from the blood circulation within 5 minutes after injection, while 42% of the liposomes containing 10% PS is still present in the blood 30 minutes after injection. After 2 h also liposomes containing 10% PS have been eliminated from the blood circulation by more than 90%. When the liposomes are composed of PC and Chol only, blood disappearance is slow, as was reported before (17).

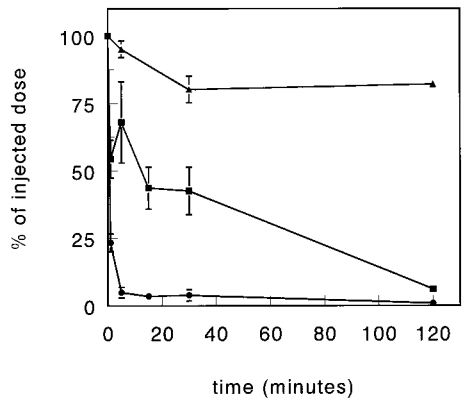

FIG. 1. Serum elimination of PC:chol (6:4) liposomes (▲), PC:chol:PS (5:4:1) liposomes (■) and PC:chol:PS (3:4:3) liposomes (●). [^{3}H]cholesteryl oleyl ether labeled liposomes were injected into rats. Blood samples were taken and radioactivity was determined as described in the methods section. Data are presented as mean ± S.E.M. of 3 to 8 rats.

Organ Distribution

Table 1 summarizes our findings on the involvement of the major organs in the elimination of liposomes from the blood, as a function of PS content. 15 min after injection almost 25% of the 10% PS-liposomes is already tightly associated with the liver; yet, nearly 40% is still in circulation at that time, while spleen uptake is low. Increasing the PS content of the liposomes to 30% and further on to 60% leads to substantial increases in blood elimination and 50% increase steps in hepatic uptake, while spleen uptake values remain at the level of a few %. Increasing PS content to a full 100% does not lead to a further increase in liver or spleen uptake. The most conspicuous changes observed 2h after injection are the considerably higher recovery of radioactivity, particularly for the 30% PS-liposomes, and the substantial difference in spleen uptake between the 10 and 30% PS-liposomes, leading to a shift in liver:spleen ratio of 2.6 for the 10%-PS liposomes to 13.6 for the 30%-PS liposomes. The improved recovery is probably related to a transient interaction of the liposomes with the hepatic endothelium during the first 10–20 min after injection, as indicated in the Discussion. The shift in organ distribution may be a reflection of a diminished affinity of the PS-rich liposomes for macrophages, as discussed below.

TABLE 1

Relative Amounts of PC:chol:PS (5:4:1 or 3:4:3 or 0:4:6 or 0:0:10) Liposomes in Blood and the Relative Uptake by the Liver and Spleen, 15 and 120 Minutes after Injection of 2 μmol Liposomal Lipid

	Blood		Spleen % of injected dose		Liver	
Liposome	15′	120′	15′	120′	15′	120′
10% PS	38.7 ± 0.3	6.1 ± 2.5	3.5 ± 0.3	19.6 ± 1.4	24.5 ± 2.5	50.8 ± 1.4
30% PS	5.7 ± 1.2	0.9 ± 0.3	2.8 ± 0.4	5.4 ± 3.6	36.5 ± 1.3	73.7 ± 1.0
60% PS	0.8 ± 0.4		3.5 ± 0.5		54.7 ± 0.7	
100% PS	2.1		2.8		44.9	

Note. When indicated, data are mean ± SD of 3 rats.

Intrahepatic Distribution

Within the liver the liposomes are, on a per cell basis, mainly taken up by the Kupffer cells and to a lesser extent by hepatocytes (Fig. 2). Liver endothelial cells, as repeatedly observed before, did not contribute significantly to the total hepatic uptake of PS-containing liposomes. No major differences between 10% and 30% PS liposomes were observed, although uptake by parenchymal cells tends to be higher for the 30% PS liposomes, at the expense of Kupffer cell uptake. Such relative reduction in macrophage affinity may also explain the relatively low uptake of the 30% PS liposomes by the spleen (Table 1), where macrophages are specifically responsible for blood elimination.

In Vitro Association with Liver Endothelial and Kupffer Cells

Kupffer cells avidly take up liposomes with 30% PS (Fig. 3A). Uptake of 10% PS-liposomes was approximately 10 fold lower and of the same order of magnitude as that of neutral liposomes without PS. In sharp contrast to the *in vivo* results endothelial cells *in vitro* were as active as the Kupffer cells in the uptake of 30% PS-liposomes (Fig. 3B). Also these

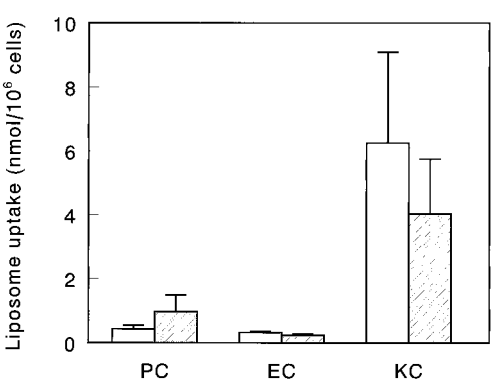

FIG. 2. Liver cell distribution of PC:chol:PS (5:4:1) liposomes (open bars) and PC:chol:PS (3:4:3) liposomes (solid bars) 2 hours after intravenous injection into rats. [^{3}H]cholesteryl oleyl ether labeled liposomes were injected into rats after which liver cells were isolated as described in the methods section. Data are presented as mean ± SD. of 3 rats. PC, parenchymal cells; EC, endothelial cells; KC, Kupffer cells.

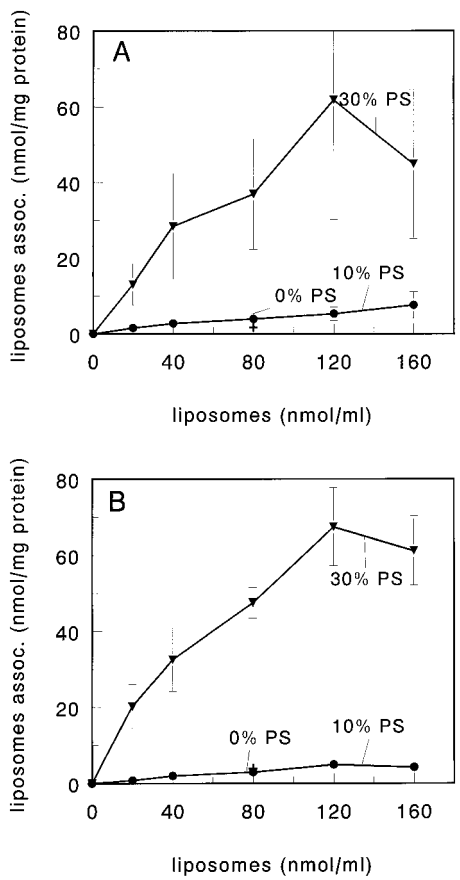

FIG. 3. Dose-dependent *in vitro* uptake of liposomes by cultured Kupffer cells (A) and endothelial cells (B) in maintenance culture. The cells were isolated and cultured as described in the methods section. The cells were incubated for 3 h at 37°C with different amounts of PC:chol:PS (5:4:1 or 3:4:3) liposomes containing a trace amount of [^{3}H]cholesteryl oleyl ether after which cell associated radioactivity was determined. PC:chol (6:4) liposomes were assayed only at a concentration of 80 nmol/ml. Data are mean ± SD of 3 experiments.

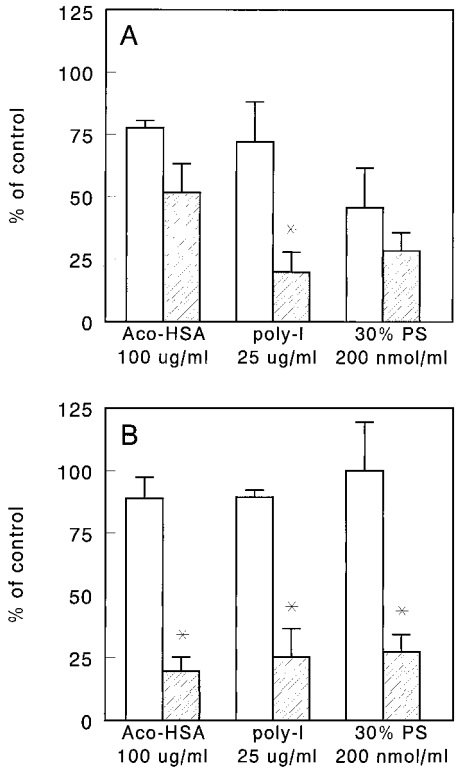

FIG. 4. Effect of Poly I, Aco-HSA and liposomes containing 30% PS on the association of PC:chol:PS (5:4:1) liposomes (open bars) and PC:chol:PS (3:4:3) liposomes (hatched bars) with Kupffer cells (A) and endothelial cells (B). The cells were incubated with 80 nmol/ml liposomes as described in the legend of Fig. 3 in the presence of the indicated compounds. Data are expressed as % of the control value without additions (mean ± S.E.M. of 3 to 6 experiments). *Indicates significantly different from liposomes containing 10% PS ($p < 0.05$). 100% values for the interaction of liposomes containing 10% PS with Kupffer and endothelial cells are 4.7 ± 1.0 and 2.9 ± 0.2 nmol of liposomes/mg of cell protein, respectively. For the interaction of liposomes containing 30% PS these values are 54.2 ± 13.5 and 44.6 ± 3.4 nmol of liposomes/mg of cell protein, respectively.

cells showed low affinity for liposomes with 0 or 10% PS.

Effect of Polyanions

Polyanions are powerful competitive inhibitors of ScR-mediated uptake processes (3). We determined the effect of polyanionic macromolecules on the interaction of the negatively charged PS-liposomes with Kupffer and endothelial cells in order to assess the involvement of this receptor in liposome uptake (Fig. 4). Aconitylated human serum albumin (Aco-HSA), polyinosinic acid (poly I) as well as unlabeled liposomes containing 30 mol% PS substantially inhibited the uptake of radiolabeled 30% PS-liposomes by liver endothelial cells to an extend of 70–80% (Fig. 4B), but no uptake of 10% PS-liposomes. The Kupffer cells showed a clearly different sensitivity towards the polyanions (Fig. 4A). Firstly, in this case both liposome types were affected in their interaction with these cells: uptake of 10% PS-liposomes was reduced by 25–50% of control values (open bars) while the uptake of 30% PS-liposomes was reduced by 50 to as much as 80% (filled bars). Secondly, of all three polyanions tested only poly I showed a significant difference in inhibitory effect between the 10 and the 30% PS-liposomes.

Effect of Sera

In order to explain the striking difference between *in vitro* and *in vivo* liposome uptake by endothelial cells as described above, we investigated the effect of sera on *in vitro* uptake conditions, so as to mimic the *in vivo* situation. Table 2 shows that at a concentration of only 10% both human serum and heat-inactivated fetal calf serum drastically suppressed the interaction of, specifically, the 30% PS-liposomes with the endothelial cells. Uptake of the 10% PS-liposomes by these cells was only partially reduced by either serum, while also the reduction of Kupffer cell uptake was only partial but identical for the two liposome types. In all cases human serum was more effective than fetal calf serum.

DISCUSSION

The mechanisms contributing to the plasma elimination of liposomes containing PS are not understood in detail, nor is that of other liposomes for that matter. In mammalian cells PS is normally localized in the inner leaflet of the cell membrane. In senescent erythrocytes, sickled erythrocytes and also in apoptotic cells the normal asymmetry of the cell membrane is no longer maintained such that these cells expose part of their PS at the surface. The presence of PS in the outer leaflet of the cell membrane is thought to function as a signal for the removal of these cells by the mononuclear

TABLE 2

Effect of 10% (v/v) Human Serum and Heat Inactivated Fetal Calf Serum on the Uptake of 80 nmol/ml PC:chol:PS (5:4:1) Liposomes and PC:chol:PS (3:4:3) Liposomes by Isolated Kupffer Cells and Endothelial Cells in Maintenance Culture

	Kupffer cells		Endothelial cells	
	10% PS	30% PS	10% PS	30% PS
None	100.0 ± 21.3	100.0 ± 24.9	100.0 ± 6.9	100.0 ± 7.6
Human serum	29.2 ± 2.2	29.3 ± 10.3	40.6 ± 9.5	5.3 ± 0.8
Fetal calf serum	70.1 ± 14.1	70.2 ± 8.5	62.7 ± 8.7	18.0 ± 2.0

Note. Experimental conditions were as described in the legend of Fig. 4. Data are expressed as the % of the control value obtained in the absence of serum (mean ± S.E.M. of 6 experiments). 100% values as in the legend of Fig. 4.

phagocyte system, possibly via specific receptors on macrophages that recognize PS (18, 19, 20). The broad ligand specificity of scavenger receptors for polyanions makes these receptors attractive candidates for mediating the binding and uptake of liposomes containing negatively charged phospholipids like PS (10). Recently, it was reported that scavenger receptors from class B, which are members of the CD36 superfamily, were able to specifically bind liposomes containing anionic phospholipids (3, 21). Our data demonstrate that, *in vivo* rat liver endothelial cells, which have been reported to possess various scavenger receptors (5, 6), do not take up liposomes containing PS to a significant extent. Recently, we showed that liver uptake of liposomes containing PS was not affected by preinjection with poly(inosinic acid), which is a well-known inhibitor of scavenger receptor mediated uptake (22). However, when the amount of PS incorporated in the liposome is increased from 10 to 30 mol% we observe a marked change in the blood clearance rate and tissue distribution. Liposomes containing 30% PS are cleared from the blood faster and accumulate to lower extents in the spleen than 10% PS-liposomes. At the same time the intrahepatic distribution of 30% PS-liposomes shifted in favor of the parenchymal cells, as compared to liposomes containing 10% PS. This is even more evident when the data from Fig. 2 are recalculated as fractions of total liver uptake. Then it appears that 75% of the 30% PS-liposomes taken up by the liver are in the parenchymal cells, while for liposomes containing 10% PS only less than half is associated with parenchymal cells. As a function of total liver uptake, the intrahepatic distribution has clearly shifted in favor of parenchymal cell uptake.

We observed a striking difference in radioactivity recovery between the 15-min and the 2-h time points. In a preliminary account of these experiments (22) we reported a 75% liver uptake of 30% PS-liposomes 15 min after injection, almost double the amount we present in Table 1. We attribute this discrepancy to a difference in the way we measured liver uptake. Initially, we measured radioactivity in homogenates of non-perfused livers, whereas in later experiments liver uptake was measured in the total liver cell suspension resulting after collagenase perfusion. Apparently, the collagenase perfusion causes loosely attached liposomes to be detached, leading to lower liver uptake values. This phenomenon is observed only at short circulation times ($\pm$15 min) and particularly with the 30% PS liposomes. We speculate that this loose and apparently transient interaction involves scavenger receptors on the endothelial cells. When cultured liver endothelial and Kupffer cells were incubated with liposomes containing 30% PS both cell types were equally active in liposome uptake. Remarkably, uptake by both endothelial and Kupffer cells of liposomes containing 10% PS was as much as 10-fold lower than that of 30% PS-liposomes. For Kupffer cells, the disproportionately high uptake of liposomes containing 30% PS compared to 10% PS-liposomes was reported by us before (23). The interaction of 30% PS-liposomes with endothelial cells could be inhibited substantially with poly anionic macromolecules, indicating that under the experimental conditions used, scavenger receptors on endothelial cells are involved in the internalization of these liposomes. Polyanions did not inhibit uptake of liposomes containing 10% PS, suggesting that, for an interaction with endothelial cell scavenger receptors, not only net negative charge but also the charge density is important. Uptake of liposomes containing 30% PS by Kupffer cells was also inhibited by poly anionic compounds, although definitely less pronounced than in the case of endothelial cells.

The strong inhibition of the interaction of 30% PS-liposomes with endothelial cells by human serum or heat inactivated fetal calf serum may offer an explanation for the remarkable difference between our *in vivo* and *in vitro* results. Presumably, plasma proteins coating the liposomes *in vivo* effectively shield the negative charges of the PS headgroup and thus prevent irreversible interaction with the scavenger receptor on endothelial cells. Furthermore plasma proteins interacting with liposomes such as complement factors and apolipoproteins are, in addition to their shielding of the liposomal surface, also actively involved in the elimination of liposomes (24). With respect to involvement of sinusoidal endothelial cells, the low uptake of 10% PS-liposomes by endothelial cells was only moderately inhibited by sera, whereas the uptake by Kupffer cells was inhibited for both liposome types to the same extent. The difference between endothelial cells and Kupffer cells in this respect, may be explained by the presence of additional uptake mechanisms, besides scavenger receptors, for PS-containing liposomes in Kupffer cells. This would also be compatible with the results obtained in the inhibition experiments.

In conclusion, the lack of liposome uptake by hepatic endothelial cells *in vivo* is not due to absence of proper receptors, but rather to a lack of recognition of the liposomes due to protein mediated shielding of the (scavenger) receptors on these cells.

ACKNOWLEDGMENTS

The authors thank Bert Dontje for excellent technical assistance with animal handling. This research was financially supported by the Netherlands Organization for Scientific Research, Grant 902-23-187.

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