Biochimica et Biophysica Acta, 1069 (1991) 273–280

BBAMEM 75404

Ricin B-chain promotes the internalisation of liposomal contents into rat hepatoma cells

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(Received 8 March 1991)

Key words: Ricin B chain; Liposome; Galactosyl binding site; Transfection; (Rat hepatoma cell)

Ricin B-chain covalently attached to liposomes has been shown to promote the binding of the liposomes to rat hepatoma cells through its galactosyl binding site. Internalisation of the bound liposomes is demonstrated by the cytotoxicity of methotrexate-containing liposomes, the transfection of cells with targeted liposomes containing pSV2-neo DNA and the intracellular activity of an enzyme encapsulated in liposomes targeted with the ricin B-chain.

Introduction

The targeting of liposomes to cells has been achieved using a variety of ligands including antibodies (for review, see Ref. 1), hormones [2] and lectins [3]. It appears from these reports that although the specific binding of liposomes to cells is readily achieved, the delivery of the liposomal contents into the cells is dependent on a variety of factors. These factors include the nature of the target site [4] the size of the liposomes [5] and the type of cell [6]. While succesful internalisation of targeted liposomes has been achieved using antibodies as the targeting agent, the same antibody does not necessarily mediate internalisation in two different cell types even if the antigen is expressed in both cells [7]. Succesful delivery of macromolecules, such as transfecting DNA, into cells has been achieved using negatively charged untargeted liposomes [8–11]; however, antibody-targeted large neutral liposomes appear to require the assistance of electroporation to deliver their contents into cells [12]. It therefore appears that apart from the use of negatively charged liposomes, which bind to most though not all cells by nature of their charge [8], there does not appear to be a widely applicable targeting agent for promoting the delivery of the contents of neutral liposomes into cells.

The wide diversity of lectins available, and their specificities for sugars, make them an interesting alternative to either antibodies or hormones as targeting agents. A variety of lectins have been used to promote the attachment of liposomes to cells [3,13,14]; however, functional delivery of the liposomal contents into the cells has not been demonstrated. In contrast to this, liposome-liposome fusion has been shown to be significantly enhanced when glycolipid containing liposomes are brought into close proximity by *Riccinus communis* agglutinin, suggesting that these lectins are able to promote interbilayer contact [15].

The B-chain of the ricin toxin (RTB) is a lectin with a specificity for galactosyl residues and is responsible for the internalisation of surface-bound ricin A-chain [16]. This ability to promote internalisation appears independent of its lectin property [16–18], and has been applied to molecules other than the ricin A-chain. For example, ricin B-chain has been conjugated to insulin, and shown to promote the internalisation and a biological effect of insulin on cells which lack the insulin receptor [19], demonstrating the ability of RTB to promote the uptake of bound molecules.

We have investigated whether the ability of the ricin B-chain to promote the internalisation of cell surface bound molecules could be applied to mediate liposome binding and internalisation. The degree of internalisation has been assessed by measuring cytotoxicity

Abbreviations: PBS, 150 mM NaCl, 10 mM Na_2HPO_4 (pH 7.4); RTB, Ricin B-chain; PC, egg yolk phosphatidylcholine; MBP-PE, *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine; BSA, bovine serum albumin.

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(methotrexate encapsulation) transfection (antibiotic resistance gene encapsulation) and the determination of a xeno-enzyme (DNase 1 encapsulation).

Materials and Methods

Lipids were obtained from Sigma except for *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (MPB-PE) which was synthesised [20].

Vesicles. Liposomes were prepared by the reverse phase evaporation procedure [21] using 9.5 μmol egg yolk phosphatidylcholine, 10 μmol cholesterol, 0.25 μmol dicetyl phosphate and 0.25 μmol MPB-PE dissolved in 1.0 ml diethyl ether. 330 μl aqueous phase containing either 100 mM calcein (Sigma), 40 mM methotrexate or pSV2-neo plasmid DNA at 1.0 mg/ml were added and either sonicated briefly, or in the case of the DNA, vortexed vigorously. DNase 1 (Boehringer, 2000 Kunitz units/mg) was encapsulated at a concentration of 1 mg/ml in PBS containing 20% glycerol using 330 μl aqueous phase with the above lipid mixture. Unentrapped marker was separated from liposomes by flotation in Ficoll (Sigma) gradients [8]. For sterilisation and to decrease size heterogeneity vesicles were subsequently extruded through sterile 0.22 μm polycarbonate membranes (Nuclepore). Crude lectins were isolated from castor bean seeds by the method of Kornfeld et al. [22], and the ricin toxin separated from ricin agglutinin by affinity chromatography on an α-L-rhamnosyl polyacrylamide column [23]. Ricin B-chain was prepared from ricin toxin by the method of Olsnes [24]. A small quantity of ricin toxin was iodinated by the lactoperoxidase method [25] prior to reduction to provide a tracer for quantifying coupling.

Coupling of ricin B-chain to vesicles. The available sulfhydryl group of the B-chain was covalently coupled [20] to the maleimido group of the MPB-PE component of the liposome. To this end 1 mg of freshly reduced ricin B-chain containing 10 μCi of ^{125}I-labeled ricin-B-chain was added to freshly prepared liposomes (10 μmol lipid in 1.0 ml PBS) and incubated at 25°C for 8 h. Liposomes were separated from non-bound protein by flotation on Ficoll gradients. Leakage of liposome contents subsequent to protein coupling was assessed by monitoring the efflux of calcein from the liposomes [26]. To monitor the extent of non-covalent binding of the protein, liposomes prepared in the absence of MPB-PE were used.

Cell binding. Liposomes prepared with 100 mM calcein in the aqueous phase were added to $2.5 \cdot 10^5$ hepatoma cells in 1.0 ml phosphate-buffered saline (PBS: 150 mM NaCl, 10 mM Na_2HPO_4 (pH 7.4)) at either 4°C or 37°C and incubated for 1 h after which the cells were washed three times with PBS and fluorescence determined either by fluorescence microscopy or by lysing the cells with 1% Triton X-100 and spectrofluorometric determination.

Electron microscopy. Liposomes on polycarbonate membranes or on cells were fixed in 1% osmium tetroxide in PBS for 30 min, followed by 2% glutaraldehyde, 2% formaldehyde for a further 30 min. Samples were immersed sequentially in 30%, 50%, 70%, 90% and 100% ethanol, subjected to critical point drying and covered with gold paladium. Samples were viewed on a Cambridge 200 SEM at 30° tilt.

Cytotoxicity. Hepatoma cells (HTC) were grown in Dulbecco's modification of Eagles Medium (Flow Laboratories) to mid-log phase and seeded into 24 well dishes at $5 \cdot 10^4$ cells per well and incubated at 37°C for 24 h. Liposomes or free drug were added and incubation continued for 24 h, after which 0.5 μCi [^{3}H]thymidine was added and incubation continued for 24 h. Cells were harvested onto glass fibre filters using a Titertek cell harvester (Flow laboratories, Rockville, MD) with an attachment for 24-well plates. The filters were submerged in 5% trichloroacetic acid for 5 min, washed in ethanol, dried, and counted in a scintillation counter.

Transfection. pSV2neo plasmid [27] was linearised with *Eco*R1 prior to encapsulation in liposomes. Hepatoma cells in mid-log phase were harvested with 1 mM EDTA in PBS and resuspended at 10^5 cells/ml in PBS containing 1 mM calcium and 1 mM magnesium. Liposomes or free DNA were added and the cells incubated at 37°C for 3 h, after which the cells were plated out in complete medium and incubated at 37°C. After 48 h fresh medium containing geneticin (Sigma, antibiotic concentration 400 μg/ml) was added and incubation continued for 12 days with medium being replaced every third day. The plates were then washed with PBS, fixed with 70% ethanol, stained with crystal violet, and colonies could be counted with the naked eye.

Intracellular DNase 1 activity. DNase 1, either free or in liposomes was added to hepatoma cells in suspension in 1.0 ml PBS and the cells incubated at 37°C for increasing time periods. The reaction was terminated by centrifuging the cells for 30 s and adding 200 μl phenol plus 100 μl 100 mM EDTA and vortexing. This was centrifuged at $10000 \times g$ for 10 min, the supernatant removed and washed with a further 200 μl phenol. The supernatant was then extracted with 400 μl diethyl ether and treated with 10 μg RNase which had been made free from DNase by heating to 100°C for 10 min [28]. The material was then applied to a 1% agarose gel containing ethidium bromide, electrophoresed at 150 V for 3 to 8 h and photographed under ultraviolet illumination.

Nuclei digestion. Nuclei were isolated from hepatoma cells by the method of Smith et al. [29] and digested with varying quantities of DNase 1 at 37°C for

1 h in 100 μl buffer (15 mM NaCl, 65 mM KCl, 15 mM spermine, 0.5 mM spermidine, 15 mM mercaptoethanol, 15 mM Tris (pH 7.4)). The reaction was terminated by the addition of 200 μl phenol. The procedure was then continued as above.

Results

Ricin B-chain has been reported to bind non-covalently to lipid membranes with a K_a of $14.5 \cdot 10^5$ M^{-1} [29]. We have, however, found that this interaction can be reversed by incubation of such liposome complexes with BSA. When ricin B-chain (52 μg) was non-covalently bound to liposomes under the conditions tested, and subsequently incubated with BSA (1 mg/ml) this figure became reduced to 7 μg. Thus the non-covalent association between ricin B-chain and liposomes is unlikely to survive incubation conditions in cell cultures required to measure the effect of the ricin B-chain. We have therefore coupled ricin-B-chain, employing its sulphydryl group and a maleimide substituted lipid in the surface of the liposome, covalently to the liposome. 270 μg of the ricin B-chain was thus attached, of which only 30 μg could be displaced by incubation with BSA i.e. 10% of the B-chain were non-covalently attached. Leakage of calcein from the liposomes prior to coupling of ricin B-chain was 4.5% (±1%). Subsequent to covalent coupling the leakage was 6.0% (±1%), indicating no severe perturbation of the bilayer by the ricin B-chain.

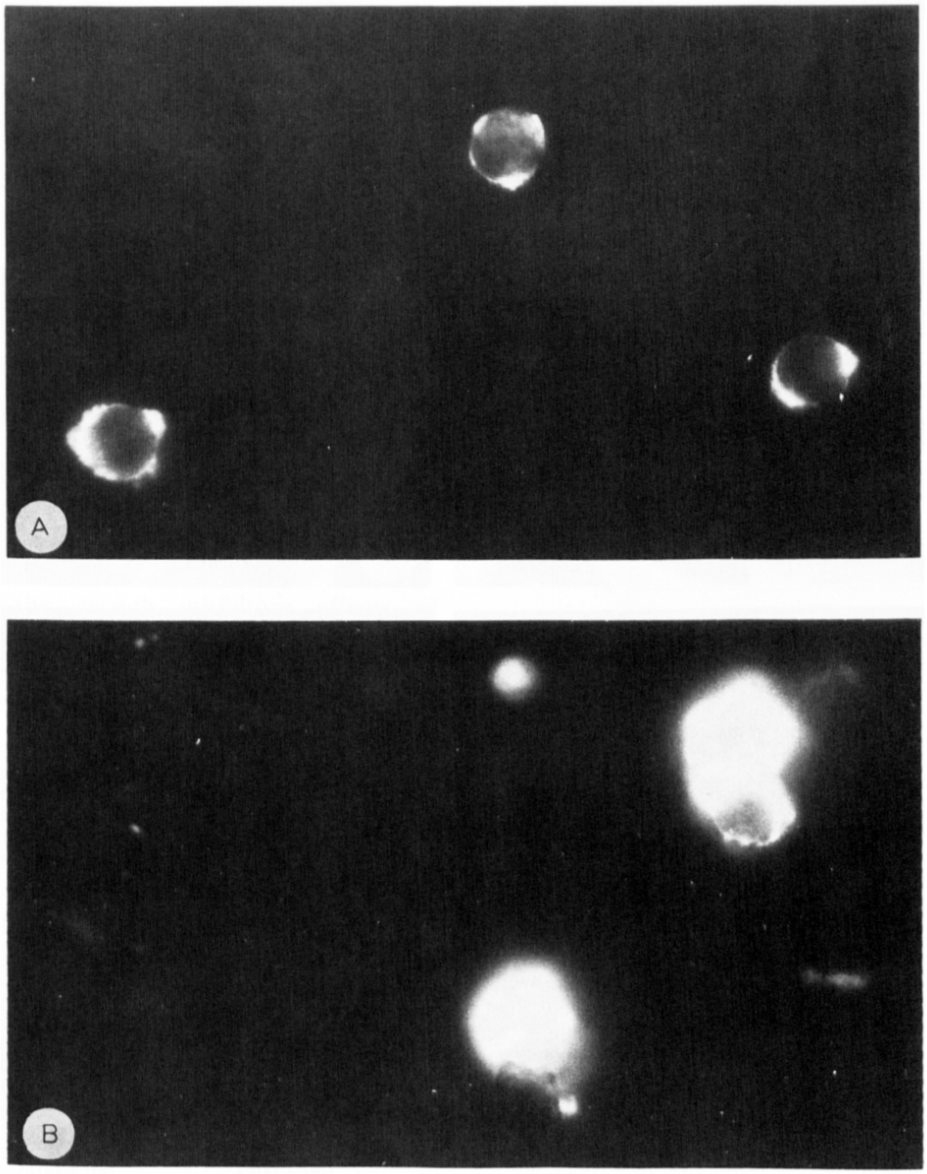


Fig. 1. Fluorescence microscopy of interaction of ricin B-chain targeted liposomes containing calcein with hepatoma cells. $2.5 \cdot 10^5$ cells in suspension in PBS were incubated with targeted liposomes corresponding to either 10 nmoles (A) or 100 nmoles (B) lipid.

When hepatoma cells were incubated with saturating (100 μM lipid) levels of targeted liposomes containing calcein, on microscopy the fluorescence was so intense that no assesment could be made of whether any of the fluorescent marker was internalised (Fig. 1B). At lower liposome concentration (10 μM lipid) the fluorescence appeared to be primarily surface bound, although some diffuse fluorescence is visible (Fig. 1A). Whether the diffuse fluorescence was due to fusion of surface bound liposomes with the cells, or arising as a result of light scattering emanating from liposomes out of the plane of focus, could not be determined. When non-targeted liposomes were incubated with the cells no fluorescence at all could be detected (results not shown).

Scanning electron microscopy of the targeted liposomes and cells (Figs. 2C, 2D) shows the presence of a large number of surface bound liposomes, whereas when untargeted liposomes are used (Fig. 2B) the cell surface appears smooth and only a few liposomes are present.

Incubating increasing quantities of targeted fluorescent liposomes with cells results in a plateau of binding being reached at 11 000 liposomes per cell (Fig. 3). The binding of untargeted liposomes amounted to only 1% of this level, therefore the lectin mediates a 100-fold increase in binding. Performing the binding in the presence of 10 mM lactose, which competes for the galactose binding site on the lectin, a significant drop in binding of liposomes to the cells occurs, indicating

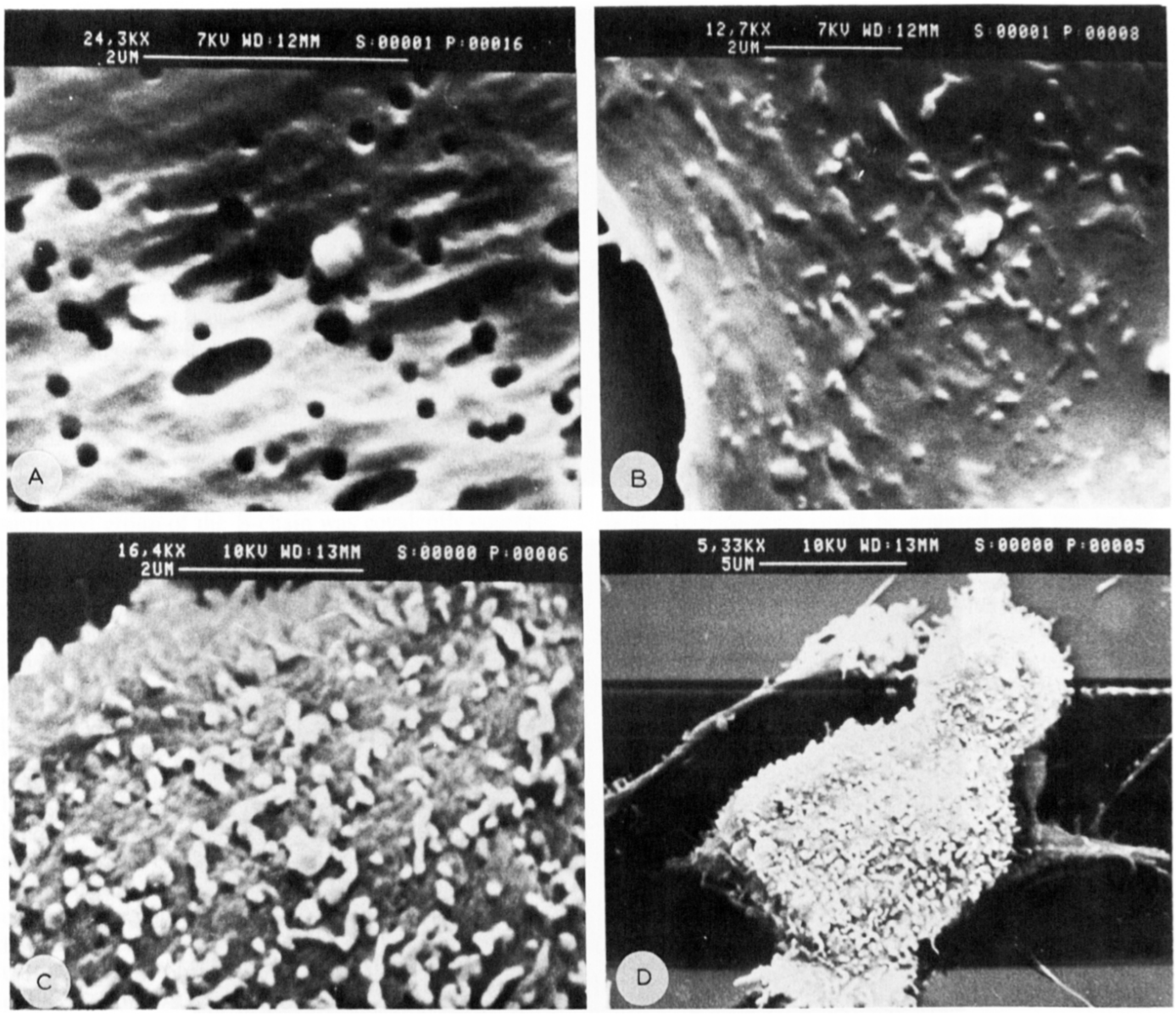


Fig. 2. Scanning electron microscopy of liposomes on 0.2 μm polycarbonate membrane (A), hepatoma cell after incubation with untargeted liposomes (B), and hepatoma cells after incubation with ricin B-chain targeted liposomes (C) and at lower magnification (D).

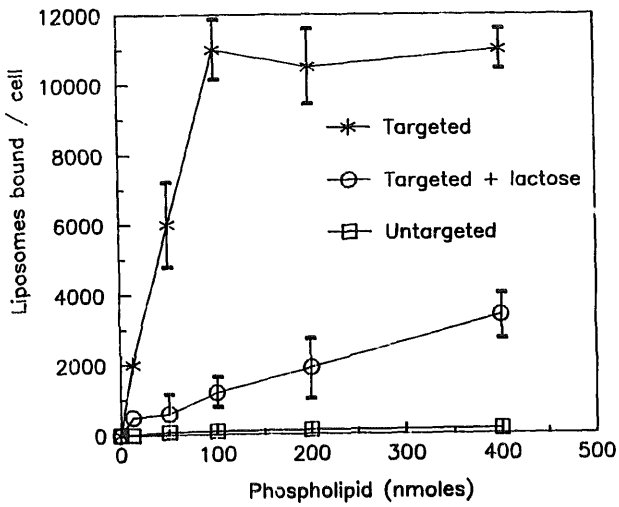


Fig. 3. Quantitation of binding of calcein containing liposomes to hepatoma cells. $2.5 \cdot 10^5$ cells in 1.0 ml PBS were incubated with increasing quantities of either RTB-targeted liposomes, RTB-targeted liposomes in the presence of 10 mM lactose, or untargeted liposomes. Incubations were performed at 4°C for 1 h after which cells were washed three times in PBS, lysed with 1% Triton X-100 and fluorescence determined.

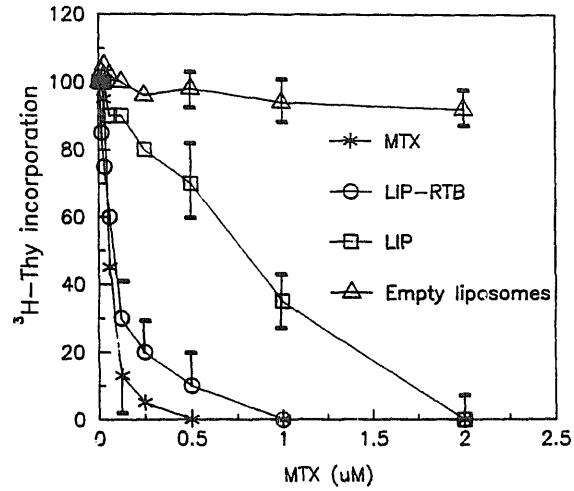


Fig. 4. Growth inhibition by methotrexate. Methotrexate either free, in RTB-targeted liposomes or in untargeted liposomes, or empty RTB-targeted liposomes were incubated at various concentrations with hepatoma cells for 24 h. [^{3}H]Thymidine incorporation into cellular DNA was assayed. For empty targeted liposomes the concentration of liposomes was based on the equivalent lipid concentration for the drug-containing liposomes.

that the binding is mediated via the sugar binding site on the lectin.

Table I shows that there is no significant change in the total binding of liposomes to cell at 4°C and at 37°C, however, the percentage of the total bound liposomes that could not be dissociated by subsequent incubation with lactose is higher if incubation is performed at 37°C. The difference represents 1300 (±241) liposomes per cell being resistant to dissociation, presumably as a result of an active process such as endocytosis, and suggests that internalisation of approximately 10% of the total bound liposomes may have taken place.

Fig. 4 shows the growth inhibitory effects of methotrexate, either as free drug, in targeted liposomes, or in untargeted liposomes. The drug entrapped in targeted liposomes exhibits an IC_{50} of 80 nM, very similar to that of the free drug, 60 nM. In contrast, when entrapped in untargeted liposomes the IC_{50} is ten fold higher at 800 nM. When empty targeted liposomes were incubated with cells no toxicity could be observed. Since free methotrexate is toxic, it could be argued that the toxicity mediated by liposomally entrapped drug is due to leakage out of the surface-bound liposomes and its subsequent entry into the cell, and not due to internalisation of the liposomes.

In order to differentiate between delivery of liposomal contents into cells, and leakage from surface bound

TABLE I

Incubation temperature dependence of lactose-mediated dissociation of ricin-B-chain targeted liposomes from hepatoma cells

Values shown are number of liposomes bound per cell after incubation of the cells with 100 nmoles targeted liposomes at 4°C or 37°C. Aliquots were either washed immediately or incubated with 50 mM lactose in PBS for 10 min at 4°C prior to washing.

	Liposomes associated per cell	
Temp.	4°C	37°C
No lactose	11000 ± 270	11500 ± 320
Lactose	2100 ± 100	3400 ± 180
% resistant	19	29

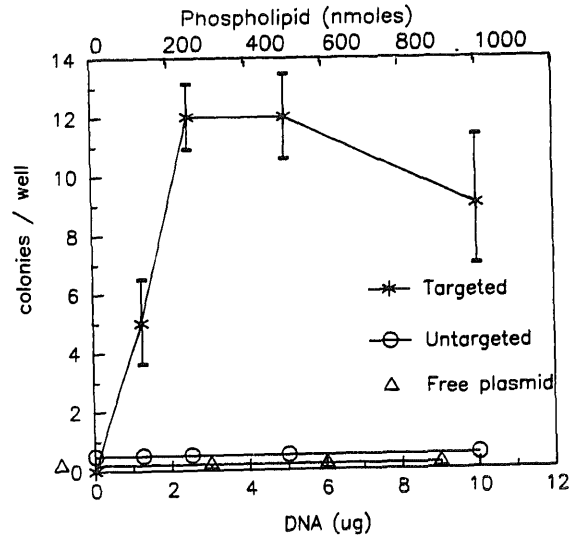
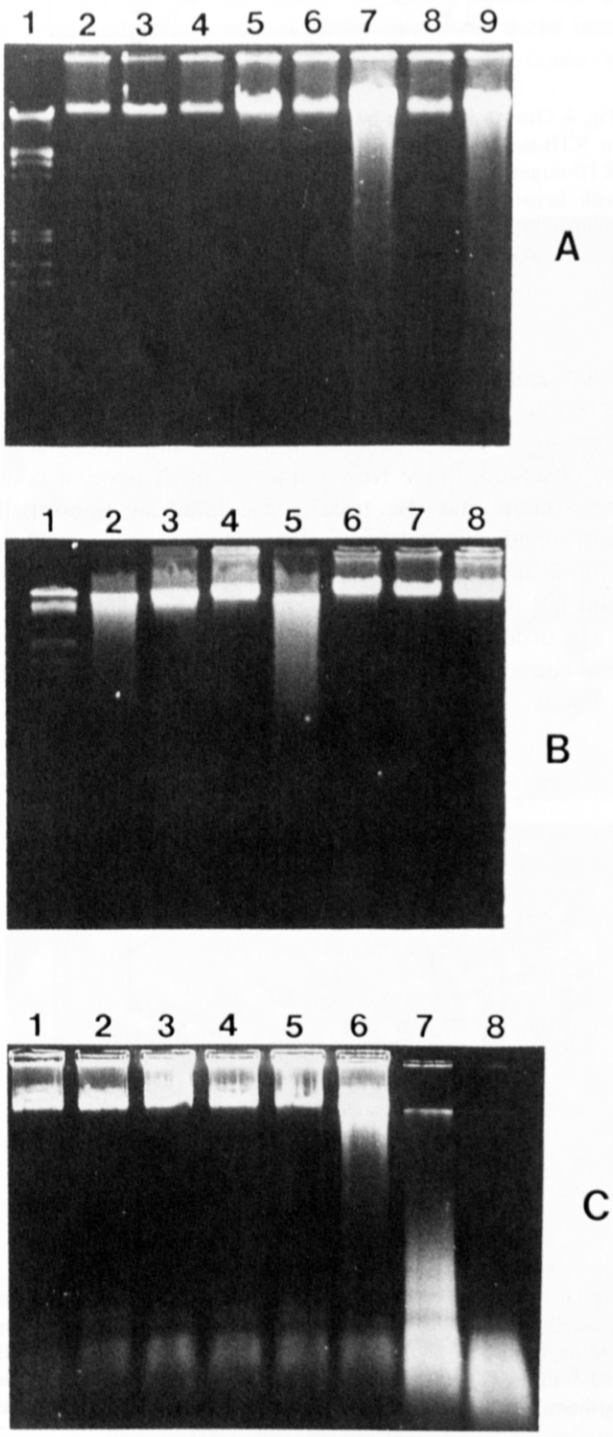


Fig. 5. Liposome mediated transfection of hepatoma cells. Liposomes, targeted with RTB or untargeted, containing pSV2neo plasmid, or free pSV2-neo plasmid were incubated with hepatoma cells and transfected cells selected for by addition of geneticin to the medium. Neither free plasmid nor plasmid in untargeted liposomes produced any transfection.

liposomes, the ability of encapsulated plasmid to transform hepatoma cells was investigated. Fig. 5 shows that pSV2neo plasmid in targeted liposomes transfected cells in a dose-dependent manner up to a maximum level of 12 transformants per $5 \cdot 10^6$ cells. Plasmid either free or in untargeted liposomes did not mediate transfection, hence the transfection by targeted liposomes demonstrates unequivocally liposome-mediated internalisation.



The target-mediated delivery of liposomal contents into cells is also obvious from the intracellular activity of encapsulated DNase 1 (Fig. 6). DNase 1 encapsulated in targeted liposomes degrades genomic DNA (Fig. 6A) whereas targeted liposomes in the presence of excess ligand or free enzymes at even 10-times higher concentration has no effect on the genomic DNA inside the cell (Fig. 6B). This demonstrates that ricin B through its galactosyl binding sites promotes the delivery of the enzymes to the nucleus (Fig. 6B). While a quantitative comparison of Figs. 6A and 6C is not possible, a qualitative assessment shows that digestion of the isolated nuclei with between 5 and 10 Kunitz units/ml of enzyme yields similar digestion to that mediated by the targeted liposomes in the same time period inside the cell. Assuming that in the digestion of isolated nuclei the concentration of enzyme in the nuclei is equal to that in the solution, then a concentration of between 5 and 10 units/ml achieves digestion similar to that experienced by the nuclei in the cells. It can then further be argued that the concentration of DNase 1 in the cells after targeting is in the order of 10 U/ml. Assuming a cell diameter of 15 μm, with a volume of $1.7 \cdot 10^{-12}$ l, 10 U/ml is equivalent to an intracellular amount of $1.7 \cdot 10^{-8}$ units of enzyme. This corresponds to between 500 and 1000 liposomes having delivered their contents into the cytoplasm within 120 min.

Discussion

It has been reported [30] that ricin B-chain binds to phospholipid vesicles with a high affinity ($K_a = 14.5 \cdot 10^5$ M^{-1}). We reasoned that this could provide a conveniant method of conjugation of the lectin to liposomes for targeting purposes. However, the conjugate is not stable under the conditions to be used to measure targeting.

Fig. 6. Effect of DNase 1 encapsulated in targeted and untargeted liposomes on intracellular genomic DNA. Liposomes containing DNase 1 were incubated under various conditions with intact cells, after which DNA was isolated and analysed on agarose gels (see Methods). (A) Time dependence of digestion after incubation of cells with free DNase 1 (lanes 2, 4, 6, 8) or liposomaly entrapped Dnase 1 (lanes 3, 5, 7, 9). DNase 1 concentration was 20 μg/ml (approx. 400 Kunitz units/ml, corresponding to 500 nmol lipid/ml). Lane 1, *Eco*R1/*Hin*d111 digested Lambda standard; Lanes 2, 3, 0 min; Lanes 4, 5, 30 min; Lanes 6, 7, 60 min; Lanes 8, 9, 120 min. (B) Digestion by DNase 1 (20 μg/ml) in targeted liposomes (lanes 2,5), targeted liposomes in presence of 50 mM lactose (lanes 3, 6), untargeted liposomes (lanes 4, 7) after incubation for 60 min (lanes 2, 3, 4) or 120 min (lanes 5, 6, 7) or free DNase 1 at 200 μg/ml. (C) Digestion of isolated hepatoma nuclei by increasing quantities of free DNase 1 at 37°C for 120 min. Lanes 1, 2, no enzyme; Lane 3, 1 unit/ml; Lane 4, 2 units/ml; Lane 5, 5 units/ml; Lane 6, 10 units/ml; Lane 7, 20 units/ml; Lane 8, 40 units/ml.

That functional targeting after covalent attachment of ricin B-chain to the liposome is taking place is shown by the almost 100-fold increase in binding, resulting in approximately 11000 liposomes binding per cell. Taking the average diameter of the liposomes to be 200 nm, and a cell diameter of 15 μm, in the order of 20000 liposomes at most could pack onto each cell. However, the influence of hydration spheres will rapidly decrease this figure, hence the observed plateau of 11000 liposomes per cell is probaly a function of the available space, and not the number of receptors. This figure is comparable to the reported [8] maximum binding obtained for negatively charged liposomes of 7000 liposomes per cell, but is achieved at a 10-fold lower lipid concentration.

Most of this binding is due to the galactosyl binding site on the lectin as demonstrated by the inhibition of binding with lactose, however, the inability of lactose to completely inhibit the binding suggests that either the affinity of binding of the multimeric complex is very high, or an alternative interaction also occurs.

Adding lactose to release lectin-specific binding after incubation at 4°C and 37°C provides a measure of the effect of temperature dependant processes on the association of the liposomes with the cells. Although the total binding is roughly the same at both temperatures, at 37°C significantly fewer of the liposomes can be dissociated, indicative of approx. 1300 liposomes per cell having dissapeared from the cell surface as the result of an active process. It is likely that internalisation is a major contributor to this, although other processes may play a role.

The order of magnitude difference in toxicity of methotrexate encapsulated in targeted and untargeted liposomes suggests that internalisation of the targeted liposomes is occurring, however, this is not unequivocal. Since free methotrexate is able to enter the cells the observed toxicity of the targeted liposomes could represent leakage of the liposomes in the vicinity of the plasma membrane, providing a locally high concentration of the drug.

Plasmids in targeted liposomes cause transfection, whereas free or in untargeted liposomes no transformation is detectable. Hence the lectin promotes the internalisation of the liposomal contents. Maximal transfection efficiency occurs at a DNA concentration of between 2 and 3 μg plasmid per well, corresponding to approx. 300 nmol lipid. However, an apparent plateau in binding occurs at 100 nmol lipid (Fig. 3), at which concentration there is insignificant transfection. This indicates that internalisation only occurs at a concentration higher than the binding plateau. This may be the result of lectin mediated binding occurring first at exposed glycoproteins distant from the plasma membrane. Only when all of the readily exposed sites have been occupied, would the less readily exposed sites, closer to the surface of the membrane, become occupied. If these sites were the ones responsible for internalisation then internalisation would only occur when the bulk of the binding sites have been occupied.

The assesment of liposomal delivery by measuring cytotoxicity is hampered by the ability of the free drug to enter the cells, and assesment by monitoring transfection is hampered by the extensive incubation periods required and the low incidence of transfection. Monitoring the activity of liposomally delivered DNase 1, circumvents these problems and yields a measure of liposomal delivery within a short time period.

It has been demonstrated that the ricin B-chain is readily linked to liposomal surfaces, and is able to mediate the binding of such targeted liposomes to galactosyl residues on cell surfaces of rat hepatoma cells with subsequent internalisation of the liposomal contents. Ricin B-chain may thus provide a convenient ligand by which to promote the delivery of liposomal contents into a wide variety of those cells exhibiting the ligand for the ricin B-chain.

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