Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 7128–7131, December 1983
Biochemistry

Targeted and nontargeted liposomes for *in vivo* transfer to rat liver cells of a plasmid containing the preproinsulin I gene

(cell separation/Southern blot analysis/β-galactose receptors)

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Communicated by Sidney Weinhouse, August 18, 1983

ABSTRACT A plasmid containing the rat preproinsulin I gene was entrapped in large liposomes and intravenously administered to rats. Four hours after inoculation, the livers were processed for the isolation of hepatocytes, Kupffer cells, and endothelial cells, DNA was purified, and the exogenous DNA was detected in the different cell DNA preparations by Southern blotting. By using liposomes consisting of phospholipids and cholesterol, Kupffer cells were shown to be, on a per cell basis, the primary target for gene incorporation. In an attempt to target the liposomes to other liver cells, a glycolipid, lactosylceramide, was included in the lipid bilayer of the liposomes; this resulted in a substantial increase in the proportion of the exogenous gene in the hepatocytes and mainly in the endothelial cells, with a simultaneous decrease of this proportion in the Kupffer cells. Thus, it is shown that inclusion of a specific glycolipid within the bilayer of the liposomes may direct the DNA-containing vesicles to specific cell types in the liver.

Liposomes have been used for *in vitro* gene transfer in eukaryotic cells in culture (1); however, the efficiency of this procedure does not exceed that of other methods, such as calcium phosphate precipitation or treatment with DEAE-dextran (2–3). Although liposomes may provide an attractive alternative with cells that are refractory to gene transfer with other methods, the greatest potentiality for liposome-mediated gene transfer seems to lie in its *in vivo* applications, in adult animals, in particular.

Intravenous (i.v.) injection to rats of a recombinant plasmid containing the rat preproinsulin I gene encapsulated in large liposomes has indeed led to the transient expression of this gene in the liver and spleen of the recipient animals (4). A significant fraction of the expressed hormone was in its physiologically active form, as demonstrated by its effect on the blood glucose level of the inoculated animals.

Hepatic uptake of large-size phospholipid–cholesterol liposomes after i.v. administration amounts to 40–60% of the injected dose and has been shown to be accounted for mainly by the Kupffer cells, to a minor extent by hepatocytes, and hardly at all by endothelial cells (5, 6). Because of the observed expression of the insulin gene *in vivo* and its transient character (4), an analysis of the intercellular location of the transported gene seemed necessary. Therefore, we have isolated hepatocytes, Kupffer cells, and endothelial cells from rat liver after i.v. injection of the liposome-entrapped rat preproinsulin I gene and assayed them for the presence of the exogenous DNA by Southern blot analysis.

Incorporation of a glycolipid with a terminal nonreducing β-galactose residue into the liposome bilayer has been reported to increase the fraction of the vesicles associating *in vivo* with hepatocytes (7–9), probably as a consequence of the interaction of the β-galactose residue with the galactose-specific lectin of the hepatocytes (10). Because Kupffer cells, the major targets of i.v. injected liposomes, may not qualify very well for the lasting expression of an exogenous gene, we extended our experiments to lactosylceramide (LacCer)-containing liposomes, in an attempt to increase gene uptake by hepatocytes.

MATERIALS AND METHODS

Recombinant DNA. Plasmid p(gR19.4) contains a 9.4-kilobase (kb) *Eco*RI fragment encoding rat preproinsulin I inserted at the *Eco*RI site of pBR322 (11). Plasmid p007 was constructed by ligating the central 2.5-kb *Bam*HI–*Hin*dIII segment of p(gR19.4), including the insulin coding sequences, to the corresponding restriction sites of pBR322. Plasmid DNAs were prepared from amplified *Escherichia coli* strain HB101 according to ref. 12.

Liposome Preparation. Liposomes consisting of egg yolk phosphatidylcholine (Sigma, purified according to ref. 13), ox brain phosphatidylserine (Sigma), and cholesterol (Sigma) [4:1:5 (mol/mol)] were prepared by reversed-phase evaporation (14). Some preparations of liposomes included a 1:6 molar ratio of dihydro-LacCer (Sigma) to phospholipids (PL). Briefly, 60 μmol of lipids dissolved in 4.5 ml of freshly distilled ether was sonicated 15 sec in a bath-type sonicator (Laboratory Supplies, Hicksville, NY) with 1.5 ml of phosphate-buffered saline containing 90 μg of DNA (including trace amounts of nick-translated [^{32}P]DNA). The ether was removed under reduced pressure in a rotary evaporator, and the liposomes were diluted to 4.5 ml with phosphate-buffered saline. Liposomes were stored at 4°C under nitrogen and were used within 1 week.

The percentage of encapsulation of the DNA was determined by treating an aliquot (200 μl) of the liposome suspensions with DNase I in the presence of 5 mM $MgCl_2$ at 37°C, followed by filtration on a Sepharose 4B column and monitoring the ^{32}P radioactivity in the excluded and retarded fractions.

One milliliter of liposomes (13 μmol of lipids) was injected in all cases into ether-anesthetized male Wistar rats (220 g) through the penis vein.

Isolation of Cell Fractions. Four hours after injection of the liposomes, hepatocytes, Kupffer cells, and endothelial cells were isolated and purified essentially as described (6). Briefly, the hepatocytes were obtained by recirculatory perfusion for about 15 min with a collagenase-containing solution (0.05%), as described by Berry and Friend (15). The perfused liver mass was

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Abbreviations: kb, kilobase(s); LacCer, lactosylceramide; PL, phospholipid(s); i.v., intravenous(ly).

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filtered through nylon gauze and the filtrate, containing mostly single cells, was freed from contaminating nonparenchymal cells by repeated low-speed centrifugations. The quality of the cells in the final suspension was assessed by their capacity to exclude trypan blue. Trypan blue exclusion (mean ± SEM) in the hepatocyte preparations used in this study was 71.5 ± 14.6%.

For the isolation of the nonparenchymal cells according to Knook and Sleÿster (16), the livers were perfused for 4 min with a 0.1% Pronase solution, removed, and cut into small pieces, which were further incubated for 60 min at 37°C in a 0.2% Pronase solution in the presence of 3 μg of DNase per ml. This results in complete digestion of the hepatocytes while Kupffer and endothelial cells remain intact. From the resulting cell suspension, the nonparenchymal cells were collected by centrifugation and the cell pellet thus obtained was resuspended and freed from hepatocyte debris and any remaining erythrocytes on a Metrizamide gradient. The purified nonparenchymal cell fraction was collected from the gradient and resolved into Kupffer and endothelial cell fractions by centrifugal elutriation in a Beckman JE-166 elutriation rotor. The two cell types could be discriminated on the basis of the peroxidase activity, which, in rats, is present in Kupffer cells and absent in endothelial cells (17). The number of peroxidase-positive cells allowed an estimation of the purity of each cell fraction. The percentages of positive cells (mean ± SEM) in the Kupffer cell fractions and in the endothelial cell fractions used in this study were 84.6 ± 5.9% and 4.9 ± 4.2%, respectively.

DNA Preparation and Southern Blot Hybridization. Cells were resuspended in 50 mM Tris·HCl, pH 7.4/150 mM NaCl/10 mM EDTA and nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (50:48:2) in the presence of 0.1% $NaDodSO_4$. DNA preparations were obtained by treatment with 10 μg of RNase A per ml and extensive dialysis against 10 mM Tris·HCl, pH 7.4/1 mM EDTA.

Restriction endonucleases were purchased from New England BioLabs or kindly supplied by L. Mallet and A. Meier (Institut Jacques Monod, Paris). Completion of the restriction digests was ensured by incubating the DNA sample with a 10-fold excess of enzyme. DNA transfer to nitrocellulose filters and hybridization conditions were performed as described (18). Final washings of the DNA filters were done at 65°C in 0.2 M NaCl/10 mM sodium phosphate, pH 6.8/1 mM EDTA. Autoradiography was performed on Fuji RX film at −70°C between Dupont Lightning Plus intensifying screens.

RESULTS

Liposome Preparations. Liposomes encapsulating the p(gR19.4) plasmid were prepared by reversed-phase evaporation and checked for their entrapment efficiency by DNase I treatment followed by filtration on Sepharose 4B. Vesicles consisting only of PL and cholesterol (PL liposomes) or also containing LacCer (LacCer liposomes) encapsulated 13.4 and 16.3% of the aqueous volume, with an entrapped volume of 6.7 and 8.1 liters/mol of PL, respectively. Electron micrographs of negatively stained liposomes showed the presence in both preparations of mostly unilamellar and oligolamellar vesicles with a size range of 0.1–1 μm. Although the preparation of the vesicles includes a short sonication step, gel electrophoresis analysis revealed only minor degradation and nicking of the plasmid DNA, with most of the plasmid remaining in the supercoiled form.

Distribution of the Exogenous p(gR19.4) Rat Insulin Plasmid Among the Different Liver Cell Types. Wistar rats were injected i.v. with 1 ml (13 μmol of total lipids or about 3 μg of encapsulated DNA) of both liposome preparations. As a control, rats were injected with 20 μg of the free rat insulin plasmid DNA. Four hours after liposome administration, livers were perfused and Kupffer cells, endothelial cells, and hepatocytes were isolated. DNA was extracted from the three cell fractions, fractionated on agarose gels following digestion with *Pst* I, and subjected to Southern blot analysis (Figs. 1–3). Because p(gR19.4), the liposome-entrapped rat insulin plasmid, contains a repeated sequence that does not allow it to be used as a hybridization probe (19), a subclone designated p007, lacking the repeated sequence, was constructed and used as the hybridization probe; the plasmid vector pBR322 was also used as a probe, thereby eliminating the background hybridization due to the endogenous rat insulin genes present in all liver cells.

By using p007 as a probe, injection of the free plasmid DNA does not result in any hybridization bands in addition to those found in normal rat liver DNA. Two hybridization bands, 5.2 and 3.0 kb, corresponding to the endogenous rat insulin I and II gene sequences, respectively, and expected to hybridize with the p007 probe (20), can be detected especially on long exposures of the autoradiograms (Fig. 3); the hybridization signal observed with the rat insulin I gene segment is the stronger of the two, because the flanking sequences present in the probe originate from this gene. An additional 8-kb hybridizing fragment is also observed on long exposures (Fig. 3) and must be due to some homology with the insulin flanking sequences present in p007, because it is not observed when using insulin cDNA probes (11, 20). With PL liposomes, three bands of 8.7, 2.1, and 1.1 kb, expected from *Pst* I cleavage of p(gR19.4) and

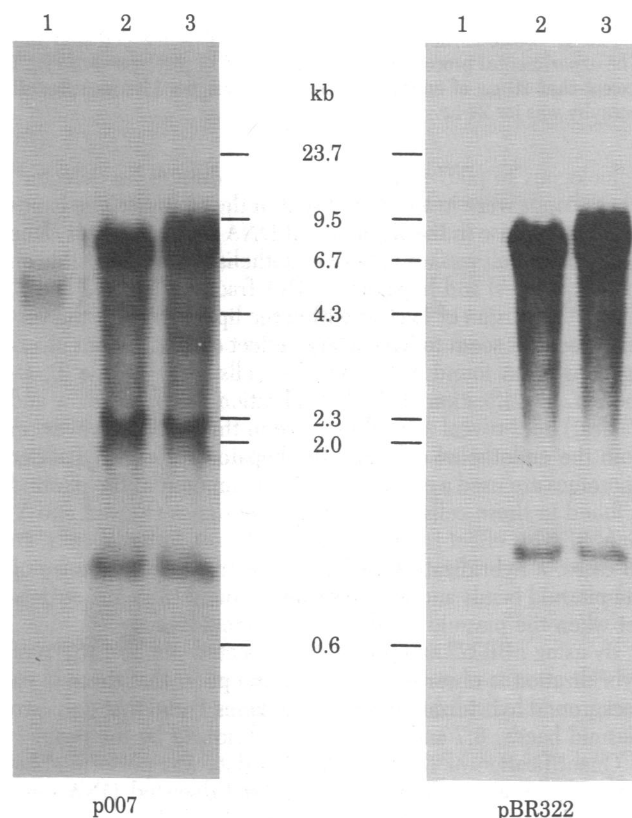

FIG. 1. Detection of the exogenous plasmid DNA in Kupffer cells. Twenty micrograms of Kupffer cell DNA from rats injected with 20 μg of free p(gR19.4) plasmid (lane 1), 3 μg of PL liposome-entrapped plasmid (lane 2), and 3 μg of LacCer liposome-entrapped plasmid (lane 3) was digested with *Pst* I, fractionated through 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized with p007 or pBR322. Autoradiography was for 24 hr. *Hind*III fragments of λ DNA were used as molecular weight markers and their lengths are indicated in kb.

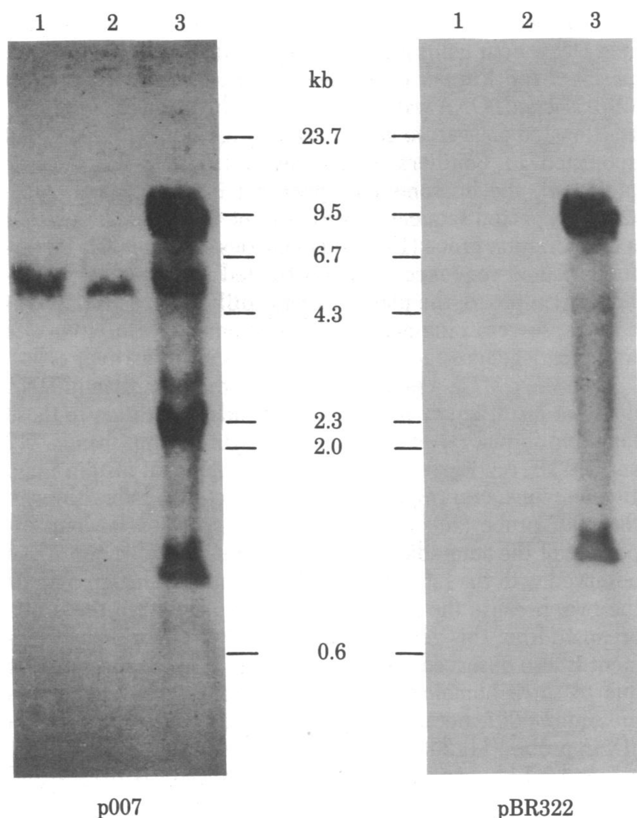

FIG. 2. Detection of the exogenous plasmid DNA in endothelial cells. The experimental procedure was as described in the legend to Fig. 1, except that 20 μg of endothelial cell DNA was used here. Autoradiography was for 24 hr.

homologous to p007 sequences, can in addition be detected. These bands were in all cases absent in the controls. The bands are most intense in the Kupffer cell DNA fraction (Fig. 1, lane 2) and are much weaker in both endothelial cell (only visible on long exposures) and hepatocyte DNA fractions (Figs. 2 and 3, lanes 2). Inclusion of LacCer within the lipid bilayer of the vesicles does not seem to have a large effect on the amount of exogenous DNA found in the Kupffer cells (Fig. 1, lane 3), although quantification of the hybridization data (see below and Table 1) does reveal a small increase in this case. However, in both the endothelial cells and the hepatocytes, when LacCer liposomes are used a considerably larger amount of the plasmid is found in these cells than with PL liposomes (Figs. 2 and 3, lanes 3). This effect is most marked in the endothelial cells. In all cases, a hybridization smear accompanied the presence of the plasmid bands and was accordingly found to be the strongest when the plasmid bands were the most intense.

By using pBR322 as a probe, the expected similar pattern of hybridization is observed, with the exception that there is no background hybridization (Figs. 1–3, lanes 1) and that only two plasmid bands, 8.7 and 1.1 kb, are hybridized by the probe.

Quantification of the hybridization data was performed by scanning the autoradiograms of the *Pst* I-digested DNA samples with a microdensitometer. The results of this analysis are shown in Table 1.

DISCUSSION

In the present work, we have examined the distribution of the liposome-encapsulated rat preproinsulin I gene among liver cells after i.v. administration. The results presented further strengthen our previous observation on the expression of exogenous genes

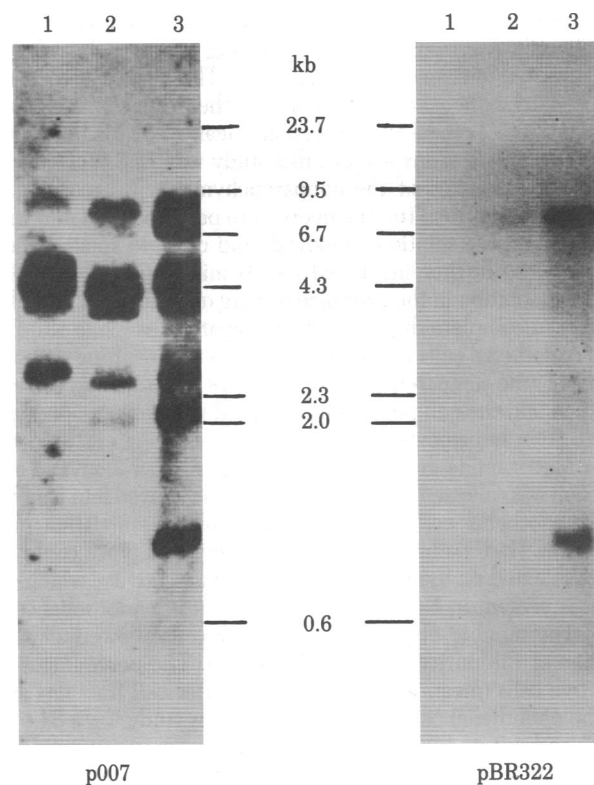

FIG. 3. Detection of the exogenous plasmid DNA in hepatocytes. The experimental procedure was as described in the legend to Fig. 1, except that 20 μg of hepatocyte DNA was used here. Autoradiography was for 12 days.

in adult animals (4, 8); they demonstrate the feasibility of gene targeting to specific cells directly *in vivo*, within the limits the liposomes can reach, and thus further extend the applications

Table 1. Distribution of the exogenous plasmid DNA among liver cell types 4 hr after liposome administration

	Plasmid copies, no. per cell*		% of plasmid†	
Cell type	PL liposomes	LacCer liposomes	PL liposomes	LacCer liposomes
Kupffer	12	16	>82	31
Endothelial	<0.2	8	<4	50
Hepatocytes	<0.2	1	<14	19

* The average number of copies of exogenous plasmid DNA per cell was determined by scanning the autoradiograms of the *Pst* I Southern blots with a Joyce–Loebl (Gateshead-on-Tyne, England) microdensitometer. In those cases in which the films were obviously saturated, scans were performed on lanes containing 5 μg of DNA instead of the usual load of 20 μg, and values were corrected accordingly. The area under all hybridization bands and smear was determined by weighing on an analytical balance. The number of copies per cell was calculated by subtracting and then dividing the observed values by those obtained for the control. The effects of different probe homologies with the endogenous rat insulin genes (assumed to exist as single copies in the rat genome; refs. 11 and 20) and with the transferred p(gR19.4) plasmid neutralize each other, because the p007 probe (6.5 kb) is roughly twice as long as the sum of the endogenous hybridizing rat insulin I sequences (2.5 kb) and rat insulin II sequences, including the first intron (0.6 kb; ref. 20), but about twice as short as the p(gR19.4) plasmid (13.7 kb). The standard deviation on these values (determined by scanning four different lanes of the same DNA samples) was found to be ±15%.

† Determined by assuming that the rat liver consists of 7% Kupffer cells, 23% endothelial cells, and 70% hepatocytes (21).

of gene transfer to eukaryotic cells.

Southern blot analysis shows that, with PL-cholesterol vesicles, the exogenous DNA is found in all liver cell fractions but in markedly different amounts. We have attempted to quantify the hybridization results by scanning the autoradiograms with a microdensitometer (Table 1); although the values we obtain are approximate values, they provide nonetheless an indication of the intrahepatic distribution of the liposome-entrapped plasmid. By using this method of quantification, it appears that with PL-cholesterol vesicles, Kupffer cells, although a minority in the liver cell population, are the major target of i.v. injected liposomes; this conclusion is in line with previous observations, in which case, labels other than DNA were used (6).

Inclusion of a LacCer in the lipid bilayer of the vesicles results in a significant change of the distribution pattern of the exogenous gene among the liver cells. Whereas with Kupffer cells only a minor increase in the amount of plasmid can be detected, a large increase is observed with both the endothelial cells and the hepatocytes. Quantification of the hybridization signals shows that, by using the "targeted" liposomes, all three cell fractions now become major targets.

The detection of the gene in the hepatocytes indicates that a fraction of the liposomes gained access to the liver parenchyma. For that to be possible, the liposomes must have crossed the endothelial lining of the sinusoid through the open fenestrations in these cells; indeed, there is a considerable overlap between the size range of the liposomes we used (0.1–1.0 μm) and that of the fenestrations (0.1–0.3 μm; ref. 22).

The presence of a β-galactosyl receptor on the surface of hepatocytes is well documented (10). Wall *et al.* (23) have shown that hepatocytes are the principal liver cells involved in the elimination of galactose-terminated glycoproteins from the blood circulation; the demonstrated function of the hepatocyte β-galactose-specific receptor in endocytosis might explain why liposomes exposing terminal nonreducing β-galactose residues are taken up much more readily than the nonglycosylated liposomes.

A galactose receptor similar to the one on hepatocytes has recently been reported on Kupffer cells (24); this might account for the small increase observed in the amount of gene taken up by these cells when using the targeted liposomes. However, the very strong increase of the uptake found in the endothelial cells is surprising, because there is no galactose-specific receptor on these cells (25). The liver endothelial cells have an endocytic activity (26, 27). Nonetheless, despite the use of the same liposome concentrations, the very different efficiency of uptake of DNA entrapped in PL liposomes as compared to LacCer liposomes by the endothelial cells might point to other uptake mechanisms than straightforward endocytosis of the injected vesicles.

Concerning the intracellular localization of the exogenous plasmid DNA, it is possible to rule out any significant binding of DNA-loaded liposomes to the cell plasma membrane; the treatment used to separate the three types of liver cells, including density-gradient centrifugations and repeated washings, would indeed remove most liposomes. Moreover, the enzymatic treatment used to prepare the cell fractions (collagenase or Pronase) would remove any adsorbed liposomes, because Pagano and Takeichi (28) have shown that treatment of the cells with trypsin releases any adsorbed liposomes, suggesting a role for cell surface proteins. Lastly, should the cell separation procedure fail to remove all liposomes, it seems unlikely that such a significant fraction of them would be bound to the plasma membrane: indeed, we detect by Southern blotting up to 4 molecules of DNA per liver cell on the average (Table 1). This may be calculated as 6% of the injected dose (75 molecules of DNA per cell), assuming that the liver of a 220-g rat consists of $1,400 \times 10^6$ cells and knowing that 50% of the liposomes are taken up by the liver (4).

Hepatocytes are secretory cells, having the enzymes required for the processing of prepolypeptides to polypeptides. This suggests that hepatocytes might qualify for the expression of liposome-transported insulin genes. The redistribution of the transported plasmid DNA among the liver cells, when changing from untargeted PL liposomes to targeted glycolipid-containing liposomes, leads to an uptake of 20% of all of the DNA recovered in the hepatocytes. This may prove to be significant for future work aiming at expression of the insulin gene in the liver in the physiologically active form of the hormone.

We thank Mieke van Galen for excellent technical assistance, Bert Dontje for help during cell isolation, and Henri Labbé for electron microscopy of liposome samples. Part of this work was supported by the Netherlands Organization for the Advancement of Pure Research (ZWO).

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