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Plant glycosides in a liposomal drug-delivery system

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Plant glycosides were incorporated into the liposomal surface to study their sugar-specific uptake by various tissues. Two steroid glycosides, namely floribundasaponin D, with rhamnose as terminal sugar, and gracillin, with glucose and rhamnose as end sugars, were selected for the purpose. ^{125}I-human IgG encapsulated liposomes composed of egg lecithin (phosphatidylcholine), cholesterol, dicetyl phosphate (optional) and either floribundasaponin D or gracillin, when injected into the tail vein of rat, showed significantly higher uptake in the rat liver than in appropriate controls. Whereas the uptake of floribundasaponin D liposomes was observed to be non-specific, the increased uptake of the gracillin liposomes, as judged from the inhibition studies with asppropriate sugars, was specific for glucose, although the receptor was unable to distinguish between the α and β anomers ('anomerically blind'). The liver-perfusion studies showed that the uptake of gracillin liposomes was mostly by non-parenchymal cells.

INTRODUCTION

The use of liposomes as a convenient drug-delivery mode has been a subject of much discussion in recent years (Gregoriadis, 1978, 1980; Stahl *et al.*, 1978. Previous work from this laboratory reported the potentiality of using glycolipids as ligands in the search for receptor sites in animal tissues (Surolia *et al.*, 1975). Neoglycoproteins having glucose, mannose and galactose residues are known to induce increased binding to liver cell membranes (Krantz *et al.*, 1976). Moreover, the terminal monosaccharide residues grafted on to the liposomal surface have been shown to play a key role in the uptake of liposomes by various organs (Ghosh & Bachhawat, 1980). The present study is concerned with the attachment of plant glycosides with different terminal sugars to the surface of liposomes in order to study their sugar-specific uptake by various organs. Two steroid glycosides, namely floribundasaponin D and gracillin, which have been isolated from Indian medicinal plants (Mahato *et al.*, 1978, 1980), were selected for the purpose. Because of the interesting structural feature of the steroid glycosides, a hydrophilic sugar moiety being attached to a hydrophobic aglycone, they are suitable for incorporation into liposomes. The hydrophilic sugar moiety remains at the surface of the liposomes, facilitating adsorption and uptake to the tissues.

MATERIALS AND METHODS

Egg lecithin (phosphatidylcholine) and cholesterol were obtained from C.S.I.R. Centre for Biochemicals, Delhi, India. Fetuin type III, human IgG, collagenase type I, dicetyl phosphate, glycogen, ovalbumin and mannan were from Sigma Chemicals, St. Louis, MO, U.S.A. Carrier-free ^{125}I-NaI was from Bhabha Atomic Research Centre, Bombay, India. 6-Carboxyfluorescein and chloramine-T were from Eastman–Kodak, Rochester, NY, U.S.A. Asialofetuin was prepared as described previously (Surolia & Bachhawat, 1977). Radioiodinated IgG (^{125}I-human IgG) was prepared by the method of Hunter (1978).

Preparation of liposomes

Liposomes were prepared as described by Gregoriadis & Ryman (1972), with phospholipid/glycoside/cholesterol/dicetyl phosphate at a molar ratio of 7:1.5:0.5:1. A thin dry film consisting of the lipids (25 mg) and the glycoside (2 mg) mixture was dispersed and sonicated for 20 min at 4 °C in 2.5 ml of phosphate-buffered saline (0.025 M-sodium phosphate/0.15 M-NaCl, pH 7.2) containing human IgG (5 mg/ml), mixed with a trace amount of ^{125}I-human IgG. Control liposomes with the aglycone were prepared exactly in the same way using phospholipid/aglycone/cholesterol/dicetyl phosphate at a molar ratio 7:1.5:0.5:1. The dicetyl phosphate was excluded for neutral liposomes. The entrapment of ^{125}I-IgG was found to be less than 10% in most of the cases. For the liposome encapsulated with 6-carboxyfluorescein, the dry film of lipid was suspended in 2.5 ml of 0.1 M dye in PBS. The dispersion as before was completed by sonication for 20 min at 4 °C under an N_2 atmosphere. Unilamellar liposomes were separated by chromatography on a column (2 cm × 25 cm) of Sepharose 4B. For gracillin liposome the presence of glucose on the liposome surface was tested by agglutination with the glucose-specific lectin concanavalin A (Surolia *et al.*, 1975). The surface-associated proteins were removed from the liposome by treatment with trypsin (0.5 mg/ml) (Shek & Sabiston, 1982).

Animal experiments

Rats of either sex (100–120 g body wt.) were used for the study. Each animal received via the tail vein an intravenous (i.v.) injection of 0.2 ml of the liposomal suspension containing approx. $(1–2) \times 10^5$ c.p.m. The

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amount of radioactivity in cardiac blood and in liver was examined at different times. To investigate the sugar-specific uptake of liposomes by the liver, different glycoproteins and polysaccharides (10 mg/100 g body wt.) were injected (i.v.) before the injection of ^{125}I-human IgG-encapsulated liposomes. In the circulation, the $t_{\frac{1}{2}}$ of liposomes with either of the glycosides was found to be 5 min. In order to test the integrity of liposomes in the presence of blood plasma, 6-carboxyfluorescein-encapsulated unilamellar liposomes were incubated *in vitro* with normal rat blood plasma for 2 h at 37 °C. No leakage was observed after 2 h of incubation. The latency of 6-carboxyfluorescein in liposomes was determined from the difference in fluorescence intensities (Aminco–Bowmann spectrofluorimeter) in the presence or absence of Triton X-100 (0.2% final concn.).

Perfusion of liver and separation of parenchymal and non-parenchymal cells

In order to examine the distribution of liposomes in different liver cell types, the cells were separated after liver perfusion by using the method of Seglen (1976) with some modifications. The livers of the experimental rats were perfused with a solution containing collagenase. A small portion (0.15–0.20 g) of the perfused liver was removed for estimation of the total uptake of liposomes by the liver. The tissue was chopped and digested in 30% (w/v) KOH solution. Digested material (1 ml) was taken for radioactivity counting. The remaining portion of the perfused liver was mildly homogenized in perfusion buffer (Dasgupta & Bachhawat, 1985) in a loosely fitting glass homogenizer using two gentle strokes to make the uniform suspension needed for the separation of parenchymal and non-parenchymal cells. The suspension, after filtration through nylon gauze (110 mesh) was kept at 4 °C for 1 h, when the parenchymal cells sedimented out, leaving the non-parenchymal cells in the supernatant. The sediment was suspended in perfusion buffer and centrifuged at 50 *g* for 2 min (Roerdink *et al.*, 1981). The process was repeated twice. The supernatants were pooled, treated with Pronase (0.5%) at 37 °C for 1 h to destroy contaminating parenchymal cells (Dasgupta & Bachhawat, 1985), and the non-parenchymal cells were pelleted down by centrifuging twice at 500 *g* for 4 min at 4 °C (Berg & Boman, 1973). The cells were counted in a haemocytometer and the cell viability, as judged by the Trypan Blue-exclusion test, exceeded 90%. Although no non-parenchymal cells could be seen in the parenchymal-cell fraction, parenchymal cells (less than 1%) were sometimes seen in non-parenchymal-cell fractions and the results were corrected accordingly. The yield of cells per g of liver was 104×10^6 parenchymal cells and 50×10^6 non-parenchymal cells. The total weight of liver was found to be approx. 3% of the total body weight. Recoveries of parenchymal and non-parenchymal cells, estimated on the basis of 450×10^6 parenchymal cells and 194×10^6 non-parenchymal cells/100 g rat (Kooistra *et al.*, 1979), were found to be 69 and 77% respectively.

RESULTS AND DISCUSSION

The rates of uptake of ^{125}I-human IgG entrapped in different types of liposomes by the liver (% of injected dose) are presented in Fig. 1. In most cases the maximum uptake was 15 min after the injection (i.v.) into the tail vein of the rats. A gradual decrease was noted in the amount of radioactivity in the liver 15 min after the injection of liposomes. The amount of uptake in the liver is dependent on the type of liposomes, as indicated in Figs. 1(*a*) and 1(*b*).

For gracillin liposomes, the uptake in the liver was found to be 72% of the injected dose for the negatively charged ones as against 65% for the neutral ones. The uptake of negatively charged control liposomes with and without the aglycone was determined to be about 46 and 42% respectively. The corresponding values for neutral liposomes, however, were found to be lower (36 and 32%). The uptake of gracillin liposomes in the

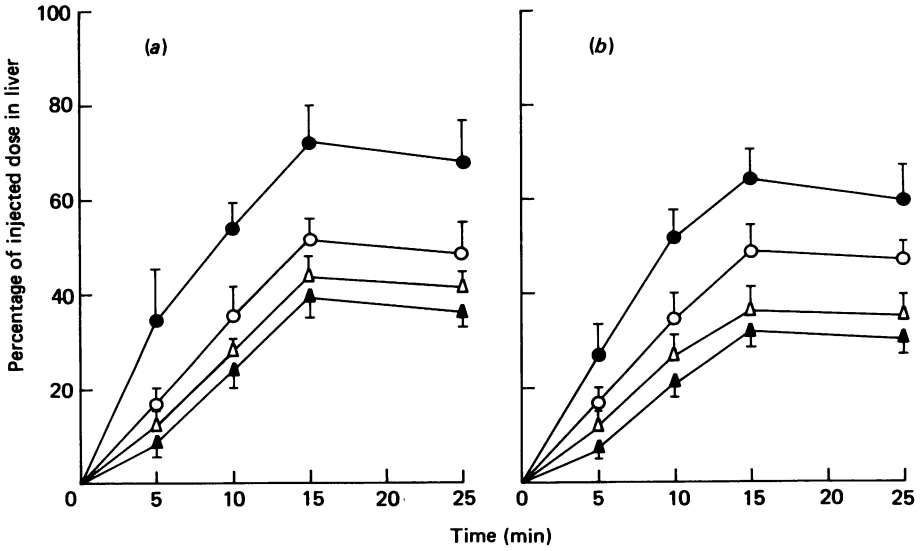

Fig. 1. Rate of uptake by liver of ^{125}I-human IgG entrapped in negatively charged (*a*) and neutral (*b*) liposomes

The different liposomes used were: ●, gracillin; ○, floribundasaponin; △, control with aglycone; and ▲, control without aglycone. The differences between the uptake of the gracillin liposomes, both negatively charged and neutral, and their corresponding controls are significant at $P < 0.001$.

presence of either glycogen (α-glucose) or cellobiose (β-glucose) was 48% for both and was almost the same as that found for the control liposomes with the aglycone. The results for uptake in the presence of glycogen or cellobiose further indicates the possible involvement of 'anomerically blind' (i.e. unable to discriminate α and β anomers) glucose receptors on the liver cell surface. Uptake was found to be unaffected (70%) when mannan (α-mannose), ovalbumin (β-mannose) or asialofetuin (galactose) was present. For floribundasaponin D liposomes, the uptake in the liver was found to be approx. 50% of the injected dose for both the negatively charged and the neutral liposomes. The uptake seemed to be unaffected when any of the three inhibitors tried (glycogen, mannan and asialofetuin) was present.

The differential uptake of ^{125}I-human IgG-entrapped liposomes by the major liver cell types 15 min after i.v. administration is shown in Table 1. The recovery of radioactivity in the cells as a percentage of that in the perfused liver was found to be approx. 85 and 70% for the negatively charged and the neutral gracillin liposomes respectively. The results show that, for gracillin liposomes, 68% of the total radioactivity incorporated in the liver was associated with the non-parenchymal-cell fraction. When the receptor sites were blocked by glycogen or cellobiose, the uptake of the non-parenchymal-cell fraction decreased and that of the parenchymal fraction increased. As judged by radioactivity measurement, followed by a cell count (Ghosh *et al.*, 1982), the uptake of ^{125}I-human IgG by non-parenchymal cells (c.p.m./10^6 cell) was three times greater than that of the parenchymal cells. With the floribundasaponin D liposomes, the uptake was similar for both the parenchymal-cell and the non-parenchymal-cell fractions, indicating the non-specific nature of the uptake.

Although there is an earlier report (Krantz *et al.*, 1976) regarding the presence of a glucose receptor in liver, the cellular localization of the receptor was not reported. The present study indicates the presence of glucose receptors (though anomerically blind) on a specific type of liver cell, namely that of non-parenchymal origin. Taking advantage of this glucose receptor it may be possible to target biologically active molecules through the glucoside-containing liposomes. This property of interaction between a cell-surface receptor and the carbohydrate moiety of the natural glycoside is of significance, and it is envisaged that glycosides of therapeutic importance having appropriate sugar molecules will be more effective than the free aglycone.

Table 1. Differential uptake of ^{125}I-human-IgG-labelled liposomes by liver cell types

The livers of the experimental rats were perfused with a solution containing collagenase. The parenchymal and non-parenchymal cells were separated by differential centrifugation. The uptake of liposomes in both of these fractions is presented as a percentage of the total radioactivity in the liver cell suspension. Results are expressed as means ± S.D. For each experiment, duplicate analyses were made and the number of independent experiments conducted is indicated in parentheses. The differences in the uptake of negatively charged gracillin liposomes with and without glycogen in both the parenchymal and non-parenchymal fractions are significant at $P < 0.001$ and $P < 0.002$ respectively.

Liposomes	Cells ...	Uptake: Parenchymal	Uptake: Non-parenchymal
Gracillin		19 ± 4 (5)	68 ± 3 (5)
Negatively charged			
+ Glycogen		40 ± 3 (4)	43 ± 5 (4)
+ Cellobiose		50 ± 4 (2)	41 ± 2 (2)
+ Ovalbumin		20 ± 5 (2)	65 ± 5 (2)
Neutral		13 ± 2 (3)	56 ± 2 (3)
Floribundasaponin D		48 ± 2 (2)	50 ± 3 (2)

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