HOMING OF LIPOSOMES TO TARGET CELLS

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SUMMARY: The possibility of homing liposomes to target cells was investigated. Liposomes containing an antitumour drug and associated with molecular probes which exhibit a specific affinity for the surface of a variety of normal and malignant cells were prepared. In vitro and in vivo experiments suggested that such probes were capable of mediating selective cellular uptake of the associated liposomes and the entrapped drug. It is anticipated that liposomes designed to home may become important tools in the control of cell behaviour.

Successful use of pharmacologically active agents in the control of cell behaviour is often dependent upon specificity and a method enabling an agent to reach its target selectively would eliminate many of the problems inherent in the conventional methods (1). Previous work has shown that allergic and other untoward reactions arising from the use of drugs, enzymes and proteins in the treatment (2,3) or prevention (4) of disease could be minimized by the entrapment of such agents in liposomes. Injected liposome-entrapped agents do not come in contact with blood and their clearance from plasma and tissue distribution is controlled by their carrier (5-7). Furthermore, the endocytic mode (8-11) of liposome uptake by cells warrants entrance of agents in otherwise inaccessible cells (12) and it also provides a convenient mechanism (13) for the liberation of entrapped agents by the disruption of liposomes in the lysosomal milieu. However, localization of injected liposomes mainly in the fixed macrophages of the liver and spleen restricts their use (1) and the suggestion has been made that direction of liposomes to alternative targets could be attained by appropriate manipulations of the liposomal surface (1).

In this report homing probes such as IgG immunoglobulins raised against a variety of cells and desialylated fetuin which binds to the parenchymal cells of the liver (14) were associated with liposomes containing bleomycin, a cytotoxic drug used in cancer chemotherapy. Our findings suggest that

liposome-associated probes can interact with target cells and selectively improve cellular uptake and interiorization of the liposomal moiety and its drug contents.

MATERIALS AND METHODS Antisera to whole cells were raised by the subcutaneous injection into New Zealand white rabbits of 7.5×10^7 HeLa cells, 2.7×10^7 AKR-A mouse leukaemia cells and 4.5×10^6 human skin fibroblasts suspended in a 1:1 (v/v) mixture of 5 mm sodium phosphate buffer pH 7.0 containing 1% NaCl (PBS) and Freund's complete adjuvant. Animals were then challenged twice intravenously at bi-weekly intervals using cells suspended in PBS and bled two weeks after the third challenge. Antisera were fractionated (15) for the preparation of the IgG fraction which was checked for purity by gel electrophoresis (16). Fractions exhibiting a single band were pooled, dialyzed against PBS, freeze-dried and subsequently analyzed for protein (17).

Entrapment in liposomes of ^{111}In-labelled bleomycin (1.1 mCi/µg Radiochemical Centre, Amersham) alone or in mixture with 3.7-25.2 mg ^{125}I-labelled (18) IgG globulin or with 100 mg ^{125}I-labelled (18) fetuin (Grand Island Biological Co., Grand Island, New York) was carried out as previously described (1). In all experiments, liposomes prepared with 40 µmoles egg lecithin, 11.4 µmoles cholesterol and 5.7 µmoles phosphatidic acid were sonicated for 1 min and in some a tracer of 5 µCi cholesteryl-[1-^{14}C]palmitate (10-20 mCi/mmol, Radiochemical Centre, Amersham) was used as a marker of the lipid phase. Of the bleomycin used, 27.4-58.4% was entrapped and of the proteins, 7.0-28.4%. Portions of the IgG-associated liposomes were adjusted to pH 8.0 with 0.1M Tris buffer and subsequently incubated with Streptomyces griseus protease (Type VI, Sigma, London, Chemical Company) (4 units/5 mg liposomal lipid) at 37°C for 24 h. Controls were treated similarly in the absence of pronase and all samples were then assayed (13) for trichloroacetic acid precipitable radioactivity: of the liposome-associated IgG 14.2-18.3% was digested with pronase. Liposomes containing bleomycin and fetuin were adjusted to pH 5.0 with 0.2M acetate buffer pH 4.5 and incubated for 5 h at 37°C in the presence of Clostridium perfringens neuraminidase (Type VI, Sigma, London, Chemical Company) (0.02 units/mg liposomal lipid) and liposomes incubated in the absence of neuraminidase served as control. Of the total sialic acid (19) in the preparation 14.0-16.5% was released upon the action of the enzyme. Elimination of pronase and neuraminidase from the preparations was carried out by molecular sieve chromatography (1). Re-chromatography of bleomycin-containing liposomes after incubation at 37°C for 5 h in the absence or presence of culture media (see below) showed that more than 92% of the radioactivity was still associated with liposomes. Liposomes suspended in 3-4 ml PBS (10.5-12.8 mg lipid/ml) were kept under oxygen-free nitrogen at 4°C until required.

In vitro experiments: Cells were cultured in Falcon bottles in Eagle's Minimal Essential Medium (MEM) containing 10% foetal calf serum and 100 i.u. ml^{-1} of penicillin and streptomycin (AKR-A cells and human skin fibroblasts) or in Eagle's Basal Medium (BME) containing 10% calf serum, 5% tryptose phosphate and antibiotics as above (HeLa cells). Exposure of cells to free or liposome-associated radiolabelled materials (0.1-0.3 ml) was carried out at 37°C. Bottles were rotated gently and at the end of the experiments media were decanted and cells washed 4 times with 10 ml PBS and detached with the addition for 5 min of 10 ml 0.25% trypsin (fibroblasts) or of 10 ml 0.05% EDTA (HeLa) cells or simply by vigorous shaking (AKR-A cells). Cells were then centrifuged at 1000 r.p.m. for 5 min, washed several times with 2 ml PBS and transferred to containers for the assay of gamma or ^{14}C radioactivity (1).

In vivo experiments: Male rats (Sprague-Dawley, weighing 100-120 g) in groups of five were injected in their tail vein with the appropriate liposome-associated radiolabelled materials and killed 15 min later. Radioactivity in

the liver was corrected for blood contamination (5).

RESULTS

Interaction of cells with IgG-carrying liposomes: As with free IgG which exhibited considerable specificity for the cell types against which it was raised (Table 1), uptake of liposome-associated IgG by any of the cell types was highest when the IgG corresponded to the cell studied: of the liposome-entrapped immunoglobulins, 0.32% anti-fibroblasts IgG was associated with fibroblasts, 1.41% anti-HeLa cells IgG with HeLa cells and 0.71% anti-AKR-A cells IgG with AKR-A cells (Fig. 1, D-F); cells exposed to their non-respective IgG associated with only 0.02-0.20% of added radioactivity (Fig. 1).

Uptake of ^{111}In radioactivity by cells exposed to liposomes containing ^{111}In-labelled bleomycin was most pronounced when liposomes were associated with the antibody corresponding to the cells studied. For instance, 0.91%

TABLE 1. UPTAKE OF ANTI-CELL ^{125}I-LABELLED IgG BY CELLS

	Preparation	Fibroblasts	HeLa cells	AKR-A cells
		Radioactivity (% of added per bottle)*		
A	IgG	0.22 ± 0.01	0.19 ± 0.01	0.41 ± 0.02
B	IgGf	1.95 ± 0.11	0.15 ± 0.02	-
C	IgGh	0.53 ± 0.02	2.10 ± 0.10	0.35 ± 0.01
D	IgGa	0.29 ± 0.20	0.16 ± 0.02	3.20 ± 0.23

Human skin fibroblasts (f) (10^7 cells/bottle), HeLa cells (h) (1.2×10^7 cells/bottle) and AKR-A mouse leukaemia cells (a) (5×10^6 cells/bottle) were incubated at 37°C for 6 h in the presence of media containing ^{125}I-labelled IgG (250-290 µg protein and $8-9 \times 10^5$ c.p.m.) from non-immune rabbits (IgG), rabbits immunized with human skin fibroblasts (IgGf), with HeLa cells (IgGh) or with AKR-A cells (IgGa) (A-D). Most of the reacted radioactivity represented non-specific IgG because in two experiments, after reincubation with fresh HeLa cells of media containing IgGh and already exposed to HeLa cells as in C, only 0.29 and 0.35% of added radioactivity was associated with cells. All differences in ^{111}In and ^{125}I radioactivities between homing and non-homing IgG immunoglobulins for each of the cell types were statistically significant ($P < 0.001$-0.05).
*Mean ± standard error. Five bottles were used in each of the experiments.

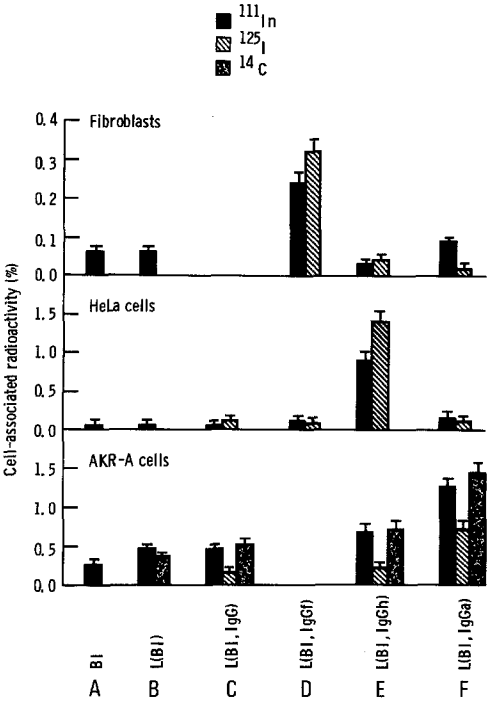

Fig. 1. Homing of liposomes by IgG immunoglobulins
Cells were exposed to media containing a trace of ^{111}In-labelled bleomycin (9 x 10^5-2.2 x 10^6 c.p.m.) free (Bl) or entrapped in liposomes (1.5-1.9 mg lipid and 3 x 10^5 d.p.m. cholesteryl ^{14}C-palmitate radioactivity when appropriate) containing: PBS, (L,Bl); IgG, (L(Bl,IgG)); IgGf, (L(Bl,IgGf)); IgGh, (L(Bl,IgGh)); IgGa, (L(Bl,IgGa)) (see legend to the table). Six bottles were used in each experiment and values are expressed as percent (mean ± standard error) of added radioactivity associated with cells. Added liposomes contained 189-310 µg ^{125}I-labelled IgG immunoglobulins (5-7 x 10^5 c.p.m.). Pretreatment of liposomes containing bleomycin and carrying anti-HeLa cells IgG, with protease which digested the IgG available on the liposomal surface, drastically diminished uptake by HeLa cells of ^{111}In radioactivity (0.10% of added radioactivity). More than 97.5% of the ^{125}I radioactivity in the media at the end of incubation was trichloroacetic acid precipitable (13). All differences in ^{111}In, ^{125}I and ^{14}C values between homing and non-homing preparations for each of the cell types were statistically significant ($P < 0.001$-0.05).

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of added ^{111}In radioactivity entrapped in liposomes carrying anti-HeLa cells IgG was taken up by HeLa cells (Fig. 1, E) and only 0.03-0.12% of ^{111}In radioactivity from liposomes associated with non-specific, anti-fibroblasts, or anti-AKR-A cells IgG (Fig. 1, C,D,F). Such improvement on the cellular up-

take of bleomycin was not due to the formation of a bleomycin-IgG complex with the IgG moiety acting, independently of liposomes, as a carrier for bleomycin: after exposure of cells to a liposome-free mixture of bleomycin and the appropriate anti-cell IgG, cellular uptake of bleomycin was low and similar to that observed with cells exposed to bleomycin alone (e.g. Fig. 1, A). It appeared more likely that following its attachment onto the cell surface, liposomal IgG effected the uptake of the associated liposomal moiety itself (and its drug contents). Indeed when cells (AKR-A) were incubated with cholesteryl ^{14}C-palmitate-labelled liposomes, uptake of ^{14}C radioactivity (marker of the liposomal bilayers) was highest (1.40%) when liposomes carrying anti-AKR-A cells IgG were used (Fig. 1, F vs B,C,D). It also appeared that only in a limited population of such liposomes could anti-cell IgG, available on their surface, effect the association of the liposomal carrier with cells. Thus, when cholesteryl ^{14}C-palmitate-labelled liposomes containing labelled bleomycin and carrying anti-AKR-A cells labelled IgG were absorbed with AKR-A cells (e.g. Fig. 1, F) and then presented to fresh AKR-A cells, cellular uptake of all three non-adsorbed radioactivities was significantly depressed (0.17-0.28% of added radioactivity).

Investigation of the subcellular fate of liposomal bleomycin taken up by cells (HeLa) via cell-specific IgG showed that only 20.5% of the cellular ^{111}In radioactivity was bound to plasma membranes (20) the remainder, presumably interiorized through endocytosis, being recovered in the lysosome-rich particulate fraction (20).

Interaction of liver cells with desialylated fetuin-carrying liposomes: The release of sialic acid from liposomal fetuin upon exposure to neuraminidase (14.0-16.5% of the total in the preparation, see METHODS) suggests that, at least, portions of the carbohydrate chains of the protein were available to the enzyme. It is therefore conceivable that, as with free desialylated fetuin (14), exposed galactose residues could interact in vivo with the liver parenchymal cells and mediate uptake of the associated liposomal

carrier and of the entrapped bleomycin. Indeed, in experiments in which rats were injected with the appropriate liposomes (Fig. 2), hepatic uptake of neuraminidase-treated liposomes was in excess (60% of the dose for ^{111}In and 46.3% of the dose for ^{125}I, L(Bl,AF)) of that of intact liposomes (40.1% for ^{111}In and 37.2% for ^{125}I, L(Bl,F)). Such difference in hepatic values between the two groups of rats reflected the amount of radioactivity directed by desialylated fetuin to the liver parenchymal cells: blockade (14) of the cells with excess desialylated fetuin depressed the hepatic uptake of neuraminidase-treated liposomes (Fig. 2, L(Bl,AF)+AF) but had no effect on the values obtained with intact liposomes (Fig. 2, L(Bl,F)+AF).

Subcellular fractionation (21) of the liver of rats injected with intact or neuraminidase-treated liposomes revealed that in both cases most of the hepatic radioactivity (61.4-67.2% ^{111}In and 56.5-62.3% ^{125}I) was localized in the lysosome-rich fraction.

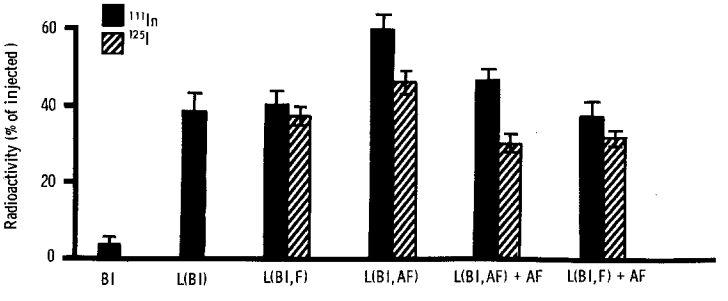

Fig. 2. Homing of liposome-entrapped bleomycin by desialylated fetuin
Rats in groups of five were injected with 1.0 ml PBS containing: a trace of ^{111}In-labelled bleomycin, (Bl) (1.5-3.0 x 10^5 c.p.m.); liposome-entrapped ^{111}In-labelled bleomycin, (L(Bl)) (2.2-2.4 mg lipid, 1.7-2.4 x 10^5 c.p.m.); liposome-entrapped ^{111}In-labelled bleomycin and ^{125}I-labelled fetuin, (L(Bl,F)) (2.3-2.6 mg lipid, 1.65-2.8 x 10^5 c.p.m. ^{111}In, 660-760 µg fetuin and 3.1-4.2 x 10^4 c.p.m. ^{125}I); L(Bl,F) incubated with neuraminidase, L(Bl,AF)); L(Bl,AF) mixed with 12 mg desialylated fetuin, L(Bl,AF)+AF); L(Bl,F) mixed with 12 mg desialylated fetuin, (L(Bl,F)+AF). Radioactivity in the total liver (mean ± standard error) is expressed as percent of the injected dose.
All differences in ^{111}In and ^{125}I radioactivities between homing and non-homing preparations were statistically significant ($P < 0.001$-0.05).

DISCUSSION

Preparation of liposomes in the presence of radiolabelled IgG or desialylated fetuin led to the association of these proteins with liposomes. Treatment of these preparations with protease (Legend to Fig. 1) or neuraminidase (see Text) revealed that some of the liposome-associated protein molecules were partly or wholly extraliposomal. Bridging of these molecules with liposomes was probably effected through the hydrophobic protein regions penetrating the lipid bilayers (22), with the active, immunologically or otherwise, portions being available for interaction with cells.

Liposome-associated homing molecules were found capable of mediating uptake of the liposomal moiety by target cells. For instance, IgG immunoglobulins raised against two human cell types (fibroblasts and HeLa cells) could discriminate between the surfaces of the respective cells (in spite of shared antigens) and this discrimination extended to liposome-associated IgG which was now effecting selective cell association of the liposomal carrier and its bleomycin contents (Fig. 1). This drug was found practically non-diffusable from liposomes (see METHODS) and, therefore, it could not have entered cells independently. Molecules other than antibodies (desialylated fetuin) were also capable of directing liposomes (Fig. 2) and it would be worth investigating whether trophic hormones or some lysosomal enzymes (23) could also serve as homing probes.

Regardless of the nature of liposome-associated homing molecules, contact of the liposome moiety with target cells will depend upon the interaction of such molecules with their respective binding sites on the cell surface. Recent evidence suggests that such interaction (e.g. binding of liposome-associated aggregated IgM to immunological determinants of phagocytes (11) or of desialylated glycoproteins to their liver cell receptors (21)) initiates endocytosis. Our data on subcellular localization of radioactivity are consistent with liposome-probe complex interiorization in the cell's lysosomes within which disruption of liposomes and liberation of entrapped agents

capable of reaching their intracellular target is then expected to occur. Indeed, it is anticipated that liposomes designed to home may become important tools in the control of cell behaviour.

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