PENETRATION OF TARGET AREAS IN THE RAT BY LIPOSOME-ASSOCIATED BLEOMYCIN, GLUCOSE OXIDASE AND INSULIN

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1. Introduction

The versatile role of liposomes as carriers of pharmacologically active agents is becoming increasingly apparent [1]. A wide variety of liposome-associated enzymes, drugs and other agents has been successfully applied in model systems for enzyme replacement therapy [2–4], cancer chemotherapy [5–7], heavy metal detoxification [8], prevention of viral infections [9] and also as immunological adjuvants [10,11], interferon inducers [12] and diagnostic agents [13]. In addition, liposomes have been used for the introduction into cells of agents which, normally, have no intracellular access [14,15]. Although it is now possible to increase selective uptake of liposomes by target cells [16], accessibility to these in vivo through capillary and other anatomical barriers can be limited by the size, charge and lipid composition of liposomes.

In the present report we have attempted to establish conditions for the passage of liposome-associated agents into areas of the body which liposomes, depending on the route of administration, can either reach only to a small extent or fail to enter altogether. As model target areas we have chosen: (a) malignant tissues approached by the intravenous route, (b) liver approached by the intraperitoneal or intramuscular route and (c) circulating blood approached by the intragastric route. In the latter case agents transported into the blood are expected to either exert their effect locally or reach, and act in, other areas.

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2. Materials and methods

Bovine brain phosphatidyl inositol (sodium salt) was purchased from Lipid Products, Nr. Redhill, Surrey, England. The source and grades of all other lipids used have been described elsewhere [2]. ^{111}In-labelled bleomycin (1.1 mCi/μg), ^{125}I-labelled insulin (> 50 μCi/μg) and ^{125}I-labelled polyvinyl-pyrrolidone (50 μCi/mg, average mol. wt. 3–4 × 10^4) were from The Radiochemical Centre, Amersham, Bucks., England. *Aspergilus niger,* glucose oxidase (grade II) from Boehringer, Mannheim, W. Germany, and insulin BP (80 units/ml) from Weddel Pharmaceuticals Ltd., London, England. Entrapment of a tracer of ^{111}In-labelled bleomycin (2.5 mCi) and of ^{125}I-labelled polyvinyl-pyrrolidone (100 μCi), of 200 mg glucose oxidase mixed with a tracer (0.4 μCi) of ^{125}I-labelled [17] glucose oxidase and of 160 units insulin mixed with 1 μCi ^{125}I-labelled insulin was carried out [16,18] in liposomes composed of egg lecithin and cholesterol (neutral, molar ratio 7:2) or the same supplemented with phosphatidic acid (negative) or stearylamine (positive, molar ratio 7:2:1). Liposomes were centrifuged at 100 000 *g* for 30 min or sonicated, when appropriate, for up to 20 min. Judging from radioactivity measurements, more than 60% of bleomycin was associated with negative liposomes and up to 5% of polyvinyl-pyrrolidone with negative, positive or neutral liposomes, 48% of glucose oxidase with negative liposomes and 20% of insulin with negative or positive liposomes. In some experiments insulin was associated (more than 90% of the 160 units used) with liposomes prepared [19] from 6 mg phosphatidyl inositol. In other experiments associa-

tion of glucose oxidase (52% of the 200 mg used) and of polyvinylpyrrolidone (5% of the tracer used) was carried out [18] in liposomes prepared with 20 and 10 mg phosphatidyl inositol respectively. With both insulin and glucose oxidase, association with this lipid led to the formation of a precipitate which could not clear upon sonication (2 min) and liposome-associated material was separated by centrifugation as above. Similarly sonicated free glucose oxidase and insulin could reduce blood glucose levels after intravenous injection into rats of 1 mg and 0.1 unit respectively and served as controls.

Tumour-bearing animals were prepared by the implantation of 2×10^6 6C HED cells per animal in the intrascapular area of C_3H mice and of 1 cm^3 Met 'A' malignant tissue per animal in the left kidney of Balb C mice. Eight (C_3H) and eleven (Balb C) days later mice were injected in their tail vein with ^{111}In-labelled bleomycin, free (2×10^6 c.p.m.) or entrapped in negative egg lecithin liposomes (2.5–3.0 mg lipid) sonicated for 0.5 min (1.5×10^6 c.p.m.) or 20 min (2.4×10^6 c.p.m.). All mice were killed 24–48 h after treatment and radioactivity, which was by then completely removed from the blood, was measured in the liver and tumour tissue. Male adult Sprague-Dawley rats weighing 100–150 g were injected intraperitoneally, or intramuscularly into the left thigh, with ^{125}I-labelled polyvinyl-pyrrolidone free (4×10^5–7×10^5 c.p.m.) or entrapped in negative egg lecithin liposomes (unsonicated or sonicated for 1 min, 2×10^5–3×10^5 c.p.m., 6.5–8.0 mg lipid). Rats were killed at time intervals and radioactivity measured in the blood and liver. Intragastric administration of ^{125}I-labelled polyvinylpyrrolidone (3×10^5–1.1×10^6 c.p.m.), glucose oxidase (175 mg/kg body weight) and insulin (480 units/kg body weight) in their free or liposome-associated form was performed in similar rats under light ether anaesthesia. Animals treated with polyvinylpyrrolidone were kept in metabolic cages, killed at time intervals and radioactivity measured in the blood, liver 24 h homogenised faeces and 24 h urine. In animals treated with glucose oxidase and insulin, blood samples obtained from their tail vein immediately before and at time intervals after treatment, were analysed for glucose [20].

3. Results and discussion

To accurately ascertain the extent to which liposomes can transport agents into tissues we adopted an approach which limits to a minimum any cell–liposomal agent association other than that mediated by the liposome carrier. To this end we employed ^{111}In-labelled bleomycin, a cytotoxic drug used in cancer chemotherapy, and ^{125}I-labelled polyvinyl-pyrrolidone both of which penetrate tissues poorly. When given in the entrapped form, these agents remain associated with the carrier in the circulation

Table 1
Uptake of radioactivity by tissues of mice injected with free and liposome-entrapped ^{111}In-labelled bleomycin

		Uptake by tissues (% ± S.D. of dose per g tissue)					
		Liver			Tumour		
Strain of mice	Implanted tumour	Free	Sonicated liposomes (0.5 min)	Sonicated liposomes (20 min)	Free	Sonicated liposomes (0.5 min)	Sonicated liposomes (20 min)
C_3H	$6C_3HED$	1.18 ± 0.25	32.8 ± 3.1	25.6 ± 1.21	1.81 ± 0.47	3.92 ± 0.51	6.82 ± 0.94
Balb C	Meth 'A'	1.36 ± 0.14	48.0 ± 7.1	19.0 ± 4.5	0.99 ± 0.39	2.38 ± 0.69	6.49 ± 1.39

$6C_3HED$ cells were implanted in the intrascapular area of 24 C_3H mice and Meth 'A' malignant tissue in the left kidney of 24 Balb C mice. Eight (C_3H) and 11 (Balb C) days later mice were injected in their tail vein with ^{111}In-labelled bleomycin, free (8 mice) or entrapped in egg lecithin negative liposomes sonicated for 0.5 or 20 min(8 mice each). All mice were killed 24–48 h after treatment. Differences in liver or tumour values between any of two groups in each of the two strains of mice were statistically significant ($P < 0.001$–0.05).

and their radiolabels persist in the site to which the agents are eventually transported by the carrier ([16] and our unpublished observations).

Uptake of radioactivity by the liver and tumour tissue of mice injected with ^{111}In-labelled bleomycin was highest when the liposome-associated drug was used (table 1). Further, uptake by tumours was greater for the smaller liposomes (20 min sonication) implying increased penetration of the vascular tumour barrier. In contrast, hepatic localisation of such liposomes was less pronounced than that of larger size liposomes (0.5 min sonication) (table 1) probably because of the decreased rate of elimination of small liposomes from the circulation [21] which allows them longer contact with, and uptake by, other areas in the body (e.g. tumour tissue).

Intraperitoneal administration of ^{125}I-labelled polyvinylpyrrolidone was followed, as evidenced by the increased hepatic values eventually attained (table 2), by the entrance of this agent into the blood in its entrapped form. Indeed, penetration of intact liposomes through the vascular membranes of the peritoneum into the circulation was possible for both large unsonicated and smaller sonicated liposomes each of which was cleared from the blood and taken up by the liver at a rate proportional to its size [21] (table 2). Following intramuscular injection, however, entrance into the blood of entrapped ^{125}I-labelled polyvinylpyrrolidone did depend on the size of the carrier. Thus, small liposomes appeared to reach the circulation and subsequently the liver while large unsonicated ones could not be traced in the blood or in the liver at 3 and 24 h (table 2). These liposomes were most probably incapable of transcapillary passage and were rapidly degraded in the site of injection with polyvinylpyrrolidone being slowly released into the blood and the lymphatics [22].

After intragastric administration of ^{125}I-labelled polyvinylpyrrolidone more than 98% of the radioactivity was recovered in the faeces in a non-dialysable form within 24 h and liver levels were very low (0.07 ± 0.02% of the dose per organ, 6 rats) at all time intervals. With polyvinylpyrrolidone entrapped in neutral, negative and positive sonicated (1.5 min) liposomes (including phosphatidyl inositol liposomes) there was a small (and similar) increase in hepatic uptake in all animals that received various liposomes and values were pooled (0.17 ± 0.02%, $P < 0.001$, 16 rats). Oral pretreatment of rats with cholestearylamine (2.5 g/kg body weight) which neutralises bile salts did not improve hepatic uptake.

Glucose oxidase, which, when given intravenously into rats (5 mg/kg body weight) was found to decrease blood glucose levels by more than 90% in 15 min, had only a minimal effect over a 24 h period when given intragastrically (175 mg/kg) in its free from (fig.1) or

Table 2
Uptake of radioactivity by the liver of rats injected with free and liposome-entrapped ^{125}I-labelled polyvinylpyrrolidone

Polyvinylpyrrolidone	Intraperitoneal injection		Intramuscular injection	
	Plasma	Liver	Plasma	Liver
Free, 3 h	31.4, 31.2	3.4, 2.4	14.8, 12.8	1.0, 0.8
24 h	16.2, 16.3	7.1, 6.7	7.5, 11.0	3.6, 5.1
Unsonicated liposomes, 3 h	5.2, 7.0	22.2, 23.1	1.6, 1.0	0.3, 0.2
24 h	3.6, 3.0	29.2, 34.6	2.2, 2.0	2.3, 0.8
Sonicated liposomes, 3 h	41.3, 34.3	14.5, 15.8	14.3, 12.0	2.4, 2.6
24 h	22.5, 23.1	25.9, 23.5	18.0, 18.8	8.6, 11.3

24 rats were injected intraperitoneally (12) and intramuscularly (12) with ^{125}I-labelled polyvinylpyrrolidone, free or entrapped in unsonicated or sonicated (1 min) egg lecithin negative liposomes and killed in pairs 3 and 24 h later. Values in the total liver (corrected for blood contamination [1] and estimated [1] total plasma from individual rats are expressed as % of the injected dose.

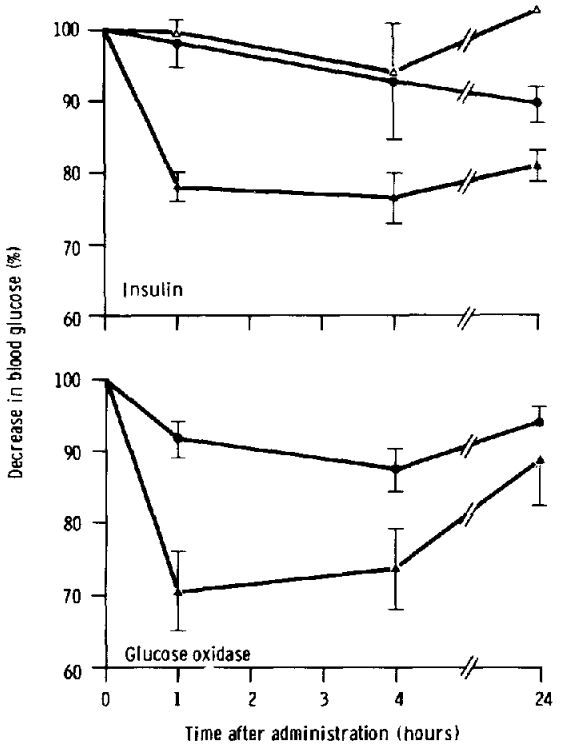

Fig.1. Blood glucose in rats given intragastrically free or liposome-associated insulin and glucose oxidase. 31 rats were given intragastrically insulin (35 units), free (7, solid circles) or associated with phosphatidyl inositol liposomes (1.8 mg lipid) (7, solid triangles); glucose oxidase (25 mg) free (7, solid circles) or associated with phosphatidyl inositol liposomes (5.0 mg lipid) (7, solid triangles); phosphatidyl inositol liposomes (5.0 mg lipid) only (3, empty triangles). Values are expressed as % ± S.E. of the blood glucose levels measured immediately before treatment in individual rats. All differences in values between free and liposome-associated proteins at 1,4 and 24 h (insulin) and 1 and 4 h (glucose oxidase) are statistically significant ($P < 0.001-0.05$).

associated with negative and positive egg lecithin liposomes and negative results were obtained with intragastrically administered insulin (480 units/kg) free (fig.1) or associated with identical liposomes. However, when glucose oxidase was given in association with phosphatidyl inositol liposomes, blood glucose levels were decreased considerably (70–73% of the values before treatment for at least 4 h thereafter, fig.1). Decrease in blood glucose (75–80% of the values before treatment) was also observed with insulin associated with phosphatidyl inositol liposomes but the effect was maintained for at least 24 h (fig.1). Phosphatidyl inositol liposomes devoid of insulin had no effect. A similar, but of unspecified potency, preparation of liposomal insulin has been claimed to produce hypoglycaemic shock in mice [19].

It appears that association of glucose oxidase and insulin with phosphatidyl inositol offers some protection to these agents from inactivation in the gut and at the same time facilitates their transport into the blood where glucose oxidase and insulin exert their effect on glucose in their respective fashion. It is not clear as yet whether entrance into the circulation of these two proteins is mediated via intact liposomes as is probably the case for the small proportion of polyvinylpyrrolidone. In contrast to the latter, glucose oxidase and insulin seem to interact with phosphatidyl inositol liposomes (see Materials and methods) and it is possible that in spite of liposome disorganisation in the gut expected to occur in the presence of bile salts and phospholipases, lipid-protein complexes enter the portal circulation through the mucous membrane of the intestines or reach the peripheral circulation through the lymphatic pathways of the gut.

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