ENTRAPMENT OF PROTEINS IN LIPOSOMES PREVENTS ALLERGIC REACTIONS IN PRE-IMMUNISED MICE

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1. Introduction

The successful use of enzymes in the treatment of inherited enzyme deficiencies and other disorders in man can be limited by several problems [1], among which immune responses to the administered, foreign to the body, enzyme are prominent. Entrapment of enzymes [2] or drugs [3] before their administration in liposomes, which are concentric lipid bilayers alternating with aqueous compartments [4], has been suggested as a means of directing therapeutic agents in isolation from the environment to diseased areas of the body. It was hoped that such a specific approach to therapy would not only circumvent possible incompatibility of administered agents with blood (e.g inactivation of some enzymes by their inhibitors in blood [5] or toxic action of some agents on blood elements) and non-specific uptake by the reticulo-endothelial system but also primary immune responses to antigenic agents. Experiments have shown that the activity of lipsome-entrapped enzymes in blood when injected into rats, retains its latency [6,7] and that such enzymes within liposomes are rapidly taken by endocytosis [8] into the liver and spleen lysosomes [6–8] where they are catabolised. The rate of degradation can be considerably decreased by cross-linking the enzyme after its entrapment in liposomes [9] and recently, enzyme-containing liposomes have been shown to alleviate a model for lysosomal storage diseases [10]. While these findings support the concept of liposomes as carriers of enzymes in the treatment of lysosomal storage diseases [1], the possibility of greatly augmenting the life span of liposomes in the blood by the adjustment of their surface charge [9] has given promising indications for the use of liposomes also as carriers of enzymes intended to act on small molecular weight substrates in blood able to diffuse through the lipid bilayers of liposomes.

In the present work the immune responses of mice injected with liposome-entrapped diphtheria toxoid were examined. It was found that although primary immune response to liposome-entrapped proteins was not abolished, occurrence of allergic reactions upon repeated challenge with the entrapped antigen was prevented.

2. Materials and methods

Diphtheria toxoid, purchased from Wellcome Research Laboratories, Beckenham, Kent, U.K., was iodinated with ^{125}I by the iodine monochloride method [11] and subsequently dialysed exhaustively against 1% NaCl. Sources and grades of phosphatidylcholine, cholesterol, stearylamine and phosphatidic acid have been described elsewhere [3]. For entrapment [3,6] in negatively charged liposomes 15.00 mg egg phosphatidylcholine, 2.20 mg cholesterol and 2.12 mg phosphatidic acid (molar ratio 7:2:1) in $CHCl_3$ were used and for entrapment in positively charged liposomes phosphatidic acid was replaced with 0.81 mg stearylamine. Following rotary evaporation of the solvent under reduced pressure, the thin lipid layer on the walls of the flask was dispersed with 2 ml ^{125}I-labelled diphtheria toxoid (12 mg/ml) adjusted to pH 7.2 with sodium phosphate buffer (final molarity 5 mM) or to pH 4.5 with sodium acetate buffer (final molarity 0.05 M) in the case of positively charged liposomes.

The suspension was then sonicated [2] for 10 sec and subsequently passed through a Sepharose 6B (Pharmacia) column (1 X 28 cm) to separate the protein-containing liposomes from the non-entrapped protein.

For immunization with diphtheria toxoid, adult mice (T. O. strain) of both sexes in groups of 5 or 6 were injected intravenously with liposome-entrapped or non-entrapped diphtheria toxoid dissolved in 5 mM sodium phosphate buffer pH 7.2 containing 1% NaCl (PBS). Two to three weeks later the mice were bled, subsequently challenged for a second time and again bled about two weeks later. Some groups of mice were challenged for a third and fourth time. Blood from the tail vein was collected in heparinised microcapillaries, centrifuged in a Microhaematocrit centrifuge (Hawksley) and the plasma assayed for the antibody titre by passive agglutination [12]. Mice previously challenged twice with non-entrapped or liposome-entrapped diphtheria toxoid were tested for the Arthus reaction: 10 μl of non-entrapped (0.01 mg) or liposome-entrapped (0.01 mg) diphtheria toxoid was injected into the right foot pad of the hind leg. The left food pad which served as control was injected with appropriate volumes of PBS. Four hr later and again at 24 and 48 hr the thickness of the foot was measured with a micrometer gauge. Other groups of mice previously challenged 2–4 times with non-entrapped or liposome-entrapped diphtheria toxoid were injected intravenously with either 0.3 ml of non-entrapped (0.40 mg) or liposome-entrapped (0.40 mg) diphtheria toxoid and observed for serum sickness. In one experiment groups of intact and immune mice were injected intravenously with non-entrapped and liposome-entrapped ^{125}I-labelled diphtheria toxoid as shown in table 3 and killed at time intervals. Radioactivity was then measured [3] in the plasma, liver, spleen and kidney.

3. Results and discussion

Entrapment of diphtheria toxoid in liposomes did not prevent the formation of antibodies in injected mice, and antibody titres were not lower than those measured in mice injected with the non-entrapped protein (see legend to table 1). Table 1 presents results from the Arthus reaction test on pre-immunised mice, expressed as the ratio of thickness of the foot pad injected with the antigen and that of the foot pad injected with PBS. It appears that regardless of the state of the antigen (entrapped or non-entrapped) with which mice were immunised, there was a moderately strong Arthus reaction (ratio 1.42) 4 hr after injection when non-entrapped diphtheria toxoid was used for the test, and no reaction, or a weak one (ratio, 1.07) when liposome-entrapped antigen was used. The absence of swelling at the sites of injection 24 or 48 hr later suggested that there was no delayed hypersensitivity. Preliminary experiments with mice immunised with non-entrapped or liposome-entrapped asparaginase suggest that with this protein too there is antibody formation despite liposomal entrapment

Table 1
Arthus reaction in immune mice

No. of mice	Challenge	Mean ratio of foot pad thickness	Range	Probability
10	Non-entrapped DT	1.42	1.05–1.83	
8	Liposome-entrapped DT	1.07	1.00–1.15	< 0.01

Mice, previously immunised by the intravenous route with non-entrapped or liposome-entrapped diptheria toxoid (DT) and with comparable concentrations of antibody against diphtheria toxoid were challenged by injection into the hind foot pad on one side of either 10 μl non-entrapped DT (0.01 mg protein) or 10 μl liposome-entrapped DT (0.01 mg protein and 0.15 mg lipid) diphtheria toxoid and on the other side by injection of 1% NaCl. Four hr later the thickness of each of the foot pads was measured and ratios calculated (see the Materials and methods section).

which, however, again inhibits the appearance of Arthus reaction.

The protection offered by the lipid bilayers to the entrapped antigen in preventing antigen-antibody interactions was further demonstrated in experiments in which mice pre-immunised with non-entrapped or liposome-entrapped diphtheria toxoid were injected intravenously with a large quantity (0.40 mg) of non-entrapped or liposome-entrapped diphtheria toxoid (table 2). Five out of seven mice injected with the non-entrapped antigen died within minutes and two were very ill but eventually recovered. All mice given the antigen entrapped in liposomes appeared normal (table 2) presumably because liposome-entrapped diphtheria toxoid did not interact with circulating antibodies. This was shown to be the case in experiments in which intact and immune mice were injected with non-entrapped and liposome-entrapped ^{125}I-labelled diphtheria toxoid and killed 20 min later (table 3). Assay of radioactivity in the blood and tissues revealed that although there was an enhanced removal of the injected non-entrapped antigen from the blood and increased localisation in the liver of immune mice, most of the diphtheria toxoid entrapped in either negatively charged or positively charged liposomes injected in such mice followed the fate of its carrier. There was no difference in radioactivity deposition in the spleen and kidneys between intact and immune mice (table 3).

Table 2
Serum sickness in immune mice

No. of mice	Challenge	Survived mice
7	Non-entrapped DT	2
7	Liposome-entrapped DT	7

Mice with comparable concentrations of antibody against diphtheria toxoid (DT) were injected in their tail vein with 0.3 ml non-entrapped DT (0.40 mg protein) or 0.3 ml liposome-entrapped DT (0.40 mg protein and 6.6 mg lipid) and were observed for serum sickness. In contrast to the survivors in the group of mice treated with non-entrapped DT, which were initially very ill, none of the mice treated with liposome-entrapped DT showed any side effects.

The present findings indicate that although entrapment in liposomes of an antigen before its injection into mice does not prevent the eventual development of an immune response, it nevertheless markedly inhibits the interaction of the antigen with its antibodies in vivo upon repeated challenge, thus preventing the development of severe, or even fatal, allergic reactions. Repeated administration of liposome-entrapped therapeutic enzymes in man may therefore prove immunologically safe.

Table 3
Fate of non-entrapped and liposome-entrapped ^{125}I-labelled diphtheria toxoid injected into immune mice

Treatment	Blood		Liver		Kidney		Spleen	
	Intact	Immune	Intact	Immune	Intact	Immune	Intact	Immune
Non-entrapped DT	9.8	3.3	24.0	54.2	2.6	2.6	2.3	2.3
	10.1	2.5	20.1	59.3	3.0	4.5	2.6	1.0
DT entrapped in negative liposomes	4.6	3.8	42.9	44.1	2.0	1.7	2.1	0.8
	6.0	4.4	40.6	42.4	2.3	1.2	1.9	3.5
DT entrapped in positive liposomes	8.5	7.1	32.1	35.1	2.6	2.8	2.4	2.2
	8.6	6.8	28.4	34.5	2.6	3.3	2.2	1.4

Intact and immune mice with comparable concentrations of antibody against diphtheria toxoid (DT) were injected in their tail vein with 0.35 ml of non-entrapped diphtheria toxoid (0.18 mg protein and 2.1×10^4 cpm) and diphtheria toxoid entrapped in negatively charged (0.18 mg protein, 2.8×10^4 cpm and 3.5 mg lipid) or positively charged liposomes (0.18 mg protein, 2.8×10^4 cpm and 3.9 mg lipid). All mice were killed 20 min after injection and radioactivity was measured in blood and tissues, corrected for blood contamination [9] and expressed as percent of the injected dose per 1 ml blood or total tissue.

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