Interaction of antibody-bearing small unilamellar liposomes with target free antigen *in vitro* and *in vivo*

Some influencing factors

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Affinity chromatography-purified and non-purified rabbit immunoglobulin G (IgG) raised against human immunoglobulin M (IgM) or kappa chain was incorporated into carboxyfluorescein-containing small unilamellar liposomes composed of egg phosphatidylcholine, cholesterol and phosphatidic acid (molar proportions 7:7:1). IgG incorporation was carried out by co-sonicating the immunoglobulin with the lipids (30% incorporated) (method A) or by interacting it with preformed liposomes bearing goat anti-(rabbit IgG) IgG (63 and 70% incorporated) (method B). (1) Judging from liposomal carboxyfluorescein-latency values, incorporation of IgG by either method did not affect liposomal stability. Furthermore, treatment of liposomes with papain released 75.1% (method A) and 93.3% and 95.1% (method B) of the IgG, suggesting that most of its antigen-recognizing Fab regions were available on the liposomal surface. This was strongly supported by the immunoelectrophoretic detection of Fab in papain-released products. (2) Liposomes bearing purified anti-IgM IgG bound 30%, (method A) and 45% (method B) of IgM in buffer. These values were about 6-fold greater (both methods) than those obtained with corresponding liposomes bearing non-purified IgG. Binding of liposomes bearing anti-(kappa chain) IgG to kappa chain in buffer was 37% of that added. In the presence of mouse blood or serum, binding of IgM to liposomes bearing purified anti-IgM IgG was decreased slightly (24 and 30% for methods A and B). However, because of the nearly complete abolition of IgM binding to liposomes bearing non-purified IgG, these values were now 20–25-fold greater than those obtained with liposomes bearing non-purified IgG. (3) In mice pre-injected with IgM, at least 36.1% and 37.7% of the antigen was bound to subsequently injected liposomes bearing anti-IgM IgG incorporated by methods A and B respectively. No binding occurred with liposomes bearing the non-purified IgG. (4) Cholesterol-rich small unilamellar liposomes bearing affinity chromatography-purified antibodies may prove useful for the specific binding of free antigens *in vivo*.

Quantitative interaction of biological targets with liposomes is often a prerequisite for their effective use in drug delivery (Gregoriadis, 1979). However, with the exception of the fixed macrophages of the reticuloendothelial system, which take up injected liposomes avidly (Gregoriadis & Ryman, 1972), most other cells, whether in culture or *in vivo*, associate with them only poorly and, in most instances, non-selectively (Gregoriadis, 1979). Work from this laboratory has shown that antibodies

against a variety of normal and malignant cells can, when incorporated into the surface of drug-containing liposomes, mediate selective association of the latter with the respective target cells (Gregoriadis & Neerunjun, 1975; Gregoriadis *et al.*, 1977). Targeting with native immunoglobulins or their F(ab')₂ dimers has now been confirmed (Magee *et al.*, 1978; Leserman *et al.*, 1980b; Torchillin *et al.*, 1980; Heath *et al.*, 1980) and extended to include aggregated immunoglobulins (Weissmann *et al.*, 1975), glycoproteins (Gregoriadis & Neerunjun, 1975; Juliano & Stamp, 1976) and glycolipids (Ghosh & Bacchawat, 1980). However, native

Abbreviations used: IgG, immunoglobulin G; IgM, immunoglobulin M.

immunoglobulins have the advantage of application to any free or cell-associated molecules against which antibodies can be raised.

In spite of convincing evidence (Gregoriadis, 1980) that appropriately modified liposomes can interact with targets selectively, information on factors that influence such interaction is scarce. For instance, in the case of liposomes coated with IgG there was no previous isolation of the cell-specific immunoglobulin species (Gregoriadis & Neerunjun, 1975; Magee *et al.*, 1978), and, when monoclonal antibodies were used (Huang *et al.*, 1980; Leserman *et al.*, 1980a), their targeting capacity was not compared with that of antibodies raised against the same target by the classical methods. Other influencing factors include stability of antibody-bearing liposomes as such or within the biological milieu, availability of the antigen-recognizing regions of antibodies on the liposomal surface and the ability of such regions to recognize and interact with their respective antigens, especially within the living animal where certain components (e.g. those of blood) are likely to interfere (Black & Gregoriadis, 1976; Krupp *et al.*, 1976; Tyrrell *et al.*, 1977; Kirby *et al.*, 1980a).

In this paper, and in the one following (Gregoriadis & Meehan, 1981), we have investigated the effect of various parameters on the interaction of antibody-bearing liposomes with target antigen in its free or cell-associated form. In the present work we incorporated non-purified and affinity chromatography-purified IgG, raised against human IgM or kappa chain, onto small unilamellar liposomes by two methods which allow different degrees of IgG availability on the liposomal surface. We found that both non-purified and purified liposomal IgG interact with free antigen in direct proportion to the availability of IgG. Such interaction is, however, far greater for the purified antibody, and occurs without significant loss of liposomal stability in the presence of whole blood or blood serum as well as in the intravascular space of animals. A related preliminary report has been presented elsewhere (Gregoriadis & Meehan, 1980).

Materials and methods

The grades and sources of egg phosphatidylcholine, sphingomyelin, cholesterol, phosphatidic acid and carboxyfluorescein have been described elsewhere (Kirby *et al.*, 1980a; Gregoriadis & Senior, 1980). Fab and Fc fragments were prepared by the method of Deutsch (1967) and anti-Fab and anti-Fc goat antisera were purchased from Miles Biochemicals. IgM was purified from the serum of a patient with Waldstrom's macroglobulinaemia by a combination of $(\text{NH}_4)_2\text{SO}_4$ precipitation, Sephadex G-200 chromatography and Pevikon block electrophoresis. Kappa chain (Bence-Jones protein)

was purified from the urine of a patient with myelomatosis by precipitation with 80%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was dialysed against 0.05 M-sodium phosphate buffer, pH 8.0, and the protein solution was passed through a DEAE column to retain most contaminating proteins (Platts-Mills & Ishizaka, 1975).

For the production of antisera against human IgM, 1 mg of the antigen in complete Freund's adjuvant was injected into the foot pads of rabbits. Animals were bled 10 days after the last of two further injections of 1 mg of antigen at 14-day intervals. Isolation (Platts-Mills & Ishizaka, 1975) of anti-IgM specific IgG were carried out through a column of Sepharose 2B (Pharmacia) linked with human IgM by the CNBr technique (Axen *et al.*, 1967). The column was washed, and anti-IgM immunoglobulins were eluted with 0.1 M-glycine buffer, pH 2.7, into 2.0 M-Tris/HCl buffer, pH 8.0. The antibodies were concentrated by negative pressure dialysis and then dialysed against boric acid/KCl/NaOH buffer, pH 8.0, containing 1% (w/v) NaCl (Platts-Mills & Ishizaka, 1975). Anti-(kappa chain) IgG was purified from rabbit antiserum raised as above against purified IgA kappa myeloma protein, using a kappa chain-Sepharose 4B column (Platts-Mills & Ishizaka, 1975). For the preparation of goat anti-(rabbit IgG) IgG, 1 mg of IgG purified (Gregoriadis & Neerunjun, 1975) from rabbit serum was injected together with complete Freund's adjuvant into the interscapular area of a goat. Two further injections of rabbit IgG were given at 14-day intervals, and 10 days later the animal was bled for the isolation (Gregoriadis & Neerunjun, 1975) of goat IgG. Non-specific rabbit IgG from the serum of intact rabbits was isolated as before (Gregoriadis & Neerunjun, 1975). Labelling of IgG with ^{131}I and of IgM and kappa chain with ^{125}I was carried out by the chloramine-T method (Klinman & Taylor, 1969).

Preparation of liposomes

Negatively charged small unilamellar liposomes were prepared as described (Kirby *et al.*, 1980a; Gregoriadis & Senior, 1980). They were composed of either egg phosphatidylcholine or sphingomyelin (20 μmol each), cholesterol and phosphatidic acid (7:7:1 molar proportion) and contained 0.2 M-carboxyfluorescein as an index of liposomal stability (Kirby *et al.*, 1980a). At this concentration the dye is fully quenched but, when liposomes become unstable, its escape into the surrounding medium and ensuing dilution enable the dye to fluoresce (Weinstein *et al.*, 1977). From values of free and total carboxyfluorescein in the sample, entrapped (latent) dye can then be estimated (see later) thus providing a means of monitoring bilayer permeability to solutes (Kirby *et al.*, 1980a). Incorporation of ^{131}I -labelled IgG (1.2–2.5 mg) in

liposomes was carried out by either of the following methods: (A) Rabbit anti-IgM or anti-(kappa chain) IgG as such, or after purification by affinity chromatography, and their respective radioiodinated derivatives were dissolved together with carboxyfluorescein (0.2M final concentration) in 2 ml of 0.1M-sodium phosphate buffer, pH 7.4, containing 1% (w/v) NaCl (phosphate buffer), and the solution was used to disrupt the dry lipids. The suspension was then sonicated (Kirby *et al.*, 1980a), centrifuged at 100 000g (r_{av} 8.6 cm) for 60 min, and the supernatant containing the small unilamellar liposomes was passed through a column (1 cm $\times$ 25 cm) of Sepharose CL 6B (Pharmacia) equilibrated with phosphate buffer to separate entrapped from non-entrapped materials. In some experiments non-specific rabbit IgG was entrapped under the same conditions. (B) Unlabelled goat IgG raised against rabbit IgG, mixed with carboxyfluorescein in phosphate buffer, was entrapped as above in liposomes which were then mixed with 0.6–1.2 mg of radioiodinated non-purified or purified rabbit anti-IgM IgG and allowed to stand at 20°C for 30 min. Liposome-bound rabbit IgG was separated from unbound rabbit IgG through a Sepharose CL 6B column (1 cm $\times$ 25 cm) equilibrated with phosphate buffer. With both methods of rabbit IgG incorporation, liposomes obtained were highly stable for several weeks, but were used within 1–2 days after their preparation. Electron microscopy studies (Kirby *et al.*, 1980a) of all liposomes showed a particle size of 30–60 nm.

Assay of carboxyfluorescein and radioactivity

Carboxyfluorescein in suitably diluted liposomal, plasma or serum samples was assayed in the absence (free dye) and presence (total dye) of Triton X-100 (1% final concentration) on a Perkin–Elmer fluorimeter with excitation and emission wavelengths of 490 and 520 nm respectively. The minimum amount of carboxyfluorescein that could be measured accurately was 4 ng/ml (Kirby *et al.*, 1980a). Latent carboxyfluorescein (index of liposomal stability) was estimated from:

$$100 \times (\text{Dye}_t - \text{Dye}_f) / \text{Dye}_t$$

where t and f denote total and free dye respectively. ^{131}I and ^{125}I radioactivities were assayed as before (Gregoriadis & Neerunjun, 1974) but when both isotopes were present, double isotope counting conditions were set to take into consideration crossover of ^{131}I into the ^{125}I channel.

Treatment of liposomes with papain

Liposomes (0.5 ml) bearing ^{131}I -labelled rabbit IgG (incorporated by either method) were mixed with 1.4 ml of 0.1M-sodium acetate buffer, pH 5.5, containing EDTA (0.002M final concn.), cysteine

(0.001M final concn.) and papain (1 mg per 100 mg of liposomal IgG). After 4–5 h incubation at 37°C, the reaction was stopped with 0.1 ml of hydroxymercuribenzoate (0.001M final concn.) and samples were passed through a Sepharose CL 6B column (1 cm $\times$ 25 cm) to separate liposome-associated from papain-released radioactivity. In control experiments, in which liposomal samples were subjected to the same procedure in the absence of papain, loss of ^{131}I radioactivity was not significant.

Immuno-electrophoresis

Products formed upon exposure of liposomes bearing anti-IgM and anti-(kappa chain) IgG to papain as described above, as well as purified Fab and Fc, were subjected to immuno-electrophoresis (Scheidtger, 1955) using anti-Fab and anti-Fc antiserum.

Experiments in vitro

Carboxyfluorescein-containing liposomes (0.5 ml) bearing ^{131}I -labelled rabbit IgG (5–8 mg of phospholipid, and 0.5–0.6 and 0.11 mg of IgG for liposomes prepared by methods A and B respectively) were mixed with equal volumes of phosphate buffer, fresh mouse (T.O. strain) heparinized whole blood or serum or fresh human serum into which 20 μg of ^{125}I -labelled IgM or ^{125}I -labelled kappa chain had been previously added and were allowed to stand at 37°C for 30 min. In other experiments carried out under identical conditions, mouse serum was replaced by 0.5 ml of increasing dilutions of human serum in phosphate buffer. Following centrifugation of the blood at 1500g (r_{av} 12 cm) for 10 min to obtain plasma, carboxyfluorescein latency was assayed in the samples, which were then passed through Sepharose CL 6B columns (1 cm $\times$ 25 cm) to separate free from liposome-bound IgM or kappa chain. Eluted fractions (28 of 1.0 ml each) were assayed for free, latent and total carboxyfluorescein and for ^{125}I and ^{131}I radioactivities.

Experiments in vivo

T.O. strain mice weighing 20–25 g were injected into the tail vein with 0.2 ml of phosphate buffer containing 60 μg of ^{125}I -labelled human IgM and 3 min later with 0.2 ml of carboxyfluorescein-containing liposomes (1.8 mg) bearing ^{131}I -labelled purified or non-purified anti-IgM IgG (0.06–0.10 mg). In control experiments, untreated mice were injected with liposomes only and, in others, mice treated with IgM were injected with phosphate buffer or free IgG. All animals were killed 5 min after the last injection, blood serum was passed through Sepharose CL 6B columns (1 cm $\times$ 25 cm) and eluted fractions (28 of 1.0 ml each) were analysed for free, latent and total carboxyfluorescein and for ^{125}I and ^{131}I radioactivities.

Results and discussion

Table 1 shows that about 30% (range 20–40%) of the rabbit or goat IgG used can be incorporated into small unilamellar liposomes when the protein is co-sonicated with the lipids (method A). This corresponds to 25–50 µg of immunoglobulin/µmol of phospholipid when 2.5 mg of the protein is used for entrapment. Entrapment values did not depend on the degree of IgG purity or animal source and are in agreement with those reported previously (Gregoriadis & Neerunjun, 1975; Huang & Kennel, 1979). Furthermore, high carboxyfluorescein latency values (97%) in such preparations (Table 1) indicate that IgG incorporation in liposomes does not affect their stability. Of the entrapped IgG, 75% (in terms of radioactivity) is released from liposomes by digestion with papain. Since it is unlikely that papain penetrates the cholesterol-rich liposomal lipid bilayer (Kirby *et al.*, 1980a) these results suggest that for most, if not all, IgG the Fab region is available on the liposomal surface. Such Fab availability was confirmed by its detection in the digestion products formed upon exposure of liposomes bearing anti-IgM and anti-(kappa chain) IgG to papain and, subsequently, immunoelectrophoresis (Fig. 1). Since there is no detectable Fc in the products (Fig. 1), this portion of IgG is apparently retained by the liposomal carrier.

Improved IgG association (63 and 70% of the IgG used, Table 1) with liposomes is achieved when the latter interact with the immunoglobulin (method B) after they have been coated by method A with goat IgG raised against rabbit IgG. Here, 93.3% and 95.1% of the liposomal rabbit IgG radioactivity is released following digestion with papain, suggesting an action of the enzyme on both the bridging goat anti-(rabbit IgG) IgG molecules and, presumably, the associated rabbit IgG. Again, judging from carboxyfluorescein latency values (97.9 and 98.1%),

the stability of liposomes that have incorporated IgG by method B remains unaffected (Table 1).

Anti-IgM IgG incorporated onto the liposomal surface by either method and anti-(kappa chain) IgG incorporated by method A are capable of interaction with IgM and kappa chain respectively without significant loss of liposomal stability (Table 2). The complex formed can then be separated (Fig. 2) from non-reacted antigen by gel filtration and can be quantified (see the Materials and methods section). According to Table 2, liposomes prepared in the presence of purified anti-IgM IgG or anti-(kappa chain) IgG (method A) bind about 30 and 37% (mean of values shown) respectively of the antigen added. For anti-IgM IgG, this value (30%) is 6-fold greater than that (about 5%) observed with liposomes coated by the same method with non-purified anti-IgM IgG. On the other hand, association of IgM with liposomes coated with a similar amount of purified anti-IgM IgG by method B is even more pronounced (about 45% of the IgM added) and again 6-fold greater than that (about 7.6%) achieved with liposomes coated (method B) with non-purified anti-IgM IgG.

Interactions between liposomal (purified) IgG and IgM also occur in the presence of mouse whole blood or serum, although their extent is diminished to approx. 24 and 30% (methods A and B respectively). Nonetheless, values are now 20–25-fold greater than when non-purified liposomal IgG is used, since the presence of whole blood or serum decreases the association of such IgG with IgM to 1.1–1.3% of that added (Table 2). It also appears that the presence of blood or serum diminishes the stability of the (purified) IgG-bearing liposomes upon interaction with antigens, albeit only modestly (87.5–89.5% latency). Such destabilization is related to the extent of interaction with the antigen; there is no loss of stability (93.8 and 96.0% latency) when liposomes bearing non-purified anti-IgM IgG in-

Table 1. *Incorporation of IgG in small unilamellar liposomes*

Carboxyfluorescein-containing liposomes were composed of egg phosphatidylcholine, cholesterol and phosphatidic acid (7:7:1 molar proportions). Incorporation of ¹³¹I-labelled IgG in liposomes was carried out by method A or method B. Stability (% latent carboxyfluorescein) of liposomes prepared in the absence of IgG was >97% (for details see the Materials and methods section). In two experiments with liposomes composed of sphingomyelin, cholesterol and phosphatidic acid (7:7:1 molar proportions), incorporated IgG was 24.1 and 30.5%, papain-released radioactivity 72.4 and 69.5% and carboxyfluorescein latency 97.5 and 98.0%. There was no significant difference in entrapment values when goat IgG was used. Numbers in parentheses denote the number of experiments; errors where shown are ±S.D.

Preparation method	Entrapped or bound rabbit IgG (%)	Papain-released radioactivity (% of total)	Liposomal stability (% latent carboxyfluorescein)
A	30.5 ± 6.9 (8)	75.1 ± 4.2 (5)	97.2 ± 1.2 (5)
B	63.0	93.3	97.0
	70.0	95.1	98.1

Fig. 1. Immunoelectrophoresis of products released after treatment of IgG-bearing liposomes with papain

Liposomes bearing anti-IgM or anti-(kappa chain) IgG were incubated with papain. Released products were then immunoelectrophoresed using anti-Fab and anti-Fc antiserum. (a) Fc standard (against anti-Fc), (b) products released from liposomes bearing anti-IgM IgG (against anti-Fc), (c) as (a), (d) products released from liposomes bearing anti-(kappa chain) IgG (against anti-Fc), (e) Fab standard (against anti-Fab), (f) products as in (b) (against anti-Fab), (g) as (e), (h) products as in (d) (against anti-Fab). Since both anti-Fab and anti-Fc cross-react with IgG, the absence of reaction in (b) and (d) suggests the absence of IgG in the papain-released products.

serum in spite of the anticipated (Black & Gregoriadis, 1976; Tyrrell *et al.*, 1977; Kirby *et al.*, 1980b; Juliano & Lin, 1980) coating of liposomes with a variety of blood plasma components. It could be, however, that such coating either does not occur when liposomes are already covered with IgG or, if it does, plasma components position themselves on liposomes in a way that affects only minimally antibody availability to, and association with, the antigen. One of the plasma components known to destabilize liposomes is high density lipoprotein (Scherphof *et al.*, 1978; Chobanian *et al.*, 1979; Kirby *et al.*, 1980b). It has been shown that such destabilization can be prevented both *in vitro* (Gregoriadis & Davis, 1979; Finkelstein & Weissmann, 1979; Kirby *et al.*, 1980a; Allen, 1981) and *in vivo* (Gregoriadis & Davis, 1979; Kirby & Gregoriadis, 1980; Kirby *et al.*, 1980a) by incorporating into the bilayers excess cholesterol. It seems (Kirby & Gregoriadis, 1980; Kirby *et al.*, 1980b) that the sterol acts by preventing the lipoproteins from removing liposomal phosphatidylcholine. Our data (Table 2) indicate that this role of cholesterol is retained in the presence of IgG in the bilayers, even during interaction with the antigens.

In further experiments we have investigated the interaction of anti-IgM IgG-bearing liposomes with IgM within the intravascular space of mice. As discussed in the legend to Fig. 2 and the Materials and methods section, after injection of such liposomes into animals previously injected with IgM, the liposomal IgG-IgM complex formed can be separated from the non-reacted IgM by molecular sieve chromatography. According to Table 3 as much as 36% and 42.8% of the administered IgM is recovered in the complex after injection of liposomes bearing anti-IgM IgG incorporated by methods A and B respectively, and none when the injected liposomes bear non-purified antibody. Indeed, association values could be even greater than those measured, since by the time of death some of the liposomal IgG-IgM complex formed may have been already removed by the liver and/or spleen (Gregoriadis *et al.*, 1977). As shown by comparing carboxyfluorescein latencies in the blood of mice injected with liposomes bearing purified or non-purified IgG there is, again (see also the experiments *in vitro*), some loss of latency (down to 80–83%; Table 3) which is apparently related to the liposomal antibody-antigen interaction. However, values for latency loss *in vivo* may have been underestimated; carboxyfluorescein liberated from liposomes has a much more rapid rate of clearance than does the dye which is still entrapped, and this can contribute to artificially high carboxyfluorescein latency values (Kirby *et al.*, 1980a). To our knowledge, this is the first clear demonstration of interaction of targeted liposomes with a biological entity in the circulating

teract with IgM under the same conditions (Table 2). As expected, interaction between 20 μ g of IgM and liposomes bearing anti-IgM IgG is totally inhibited in the presence of excess antigen in 0.5 ml of human serum (80–180 mg of IgM/ml; Clamp & Johnson, 1972) and inhibition decreases with increasing dilution of serum, thus confirming the specificity of IgM binding (Table 2 and its legend). It is of interest that these interactions of liposomal IgG with antigens occur in the presence of whole blood or

blood. In work (Campbell *et al.*, 1980) designed to alter antigen (digoxin) excretion by the use of liposomes bearing anti-digoxin antibodies, the extent of antigen-antibody binding in the blood was uncertain.

In conclusion, our findings indicate that IgG can be incorporated into small unilamellar liposomes by forming them in the presence of the antibody (method A) without loss of liposomal stability. In similar studies Huang & Kennel (1979) have observed formation of vesicle aggregates. We have performed electron microscopy on some of our sonicated preparations and found very little aggregation (G. Gregoriadis & A. Meehan, unpublished work). This discrepancy between the two studies could be attributed to different phospholipid

composition, the presence of excess cholesterol in our liposomes, or both. Regarding the mechanism by which IgG associates with liposomes during sonication, it has been suggested (Huang & Kennel, 1979) that sonication induces transient perturbation in the bilayers which then promotes partial insertion of the IgG into the bilayers. Our results (Table 1), showing considerable release of radioactivity after treatment of liposomal ^{125}I -labelled IgG with papain, support this view. Furthermore, the availability (Table 1) of the antigen-recognizing Fab for interaction with IgM or kappa chain (Table 2) and the presence of Fab and absence of Fc in the papain-released products (Fig. 1) suggest that insertion is effected through the Fc region of the immunoglobulin. On the other hand, incorporation of goat

Table 2. *Binding in vitro of IgM or kappa chain to liposomes bearing the respective antibodies*

Samples of antibody-bearing liposomes containing 0.2 M-carboxyfluorescein were mixed with equal volumes of phosphate buffer, mouse blood, mouse serum or undiluted or diluted human serum containing 20 μg of added ^{125}I -labelled human IgM [(5–12) $\times 10^4$ c.p.m.] or kappa chain (8×10^4 c.p.m.) and allowed to stand at 37°C for 30 min. Following assay of carboxyfluorescein latency, samples were chromatographed to separate free from liposome-associated antigen (Fig. 2). Values for bound antigen from two to three experiments are expressed as % of added IgM or kappa chain and have been corrected for non-specific binding occurring with liposomes bearing non-specific rabbit IgG. Non-specific binding (mean $\pm$ s.d.) was $4.7 \pm 0.2\%$ (five experiments) in the presence of phosphate buffer and $2.8 \pm 0.2\%$ (five experiments) in the presence of mouse serum. Values for liposomal stability before incubation are shown in Table 1 (for details see the Materials and methods section).

IgG in liposomes	Medium	IgM or kappa chain bound (% of added)	Liposomal stability in medium (% latent carboxyfluorescein)
Purified anti-IgM (method A)	Phosphate buffer	30.4, 30.2, 29.6	96.6, 97.0, 96.5
	Mouse blood	24.6, 24.0, 23.5	
	Mouse serum	23.0, 24.5, 26.0	88.5, 87.6
	Human serum*	0.0, 0.0	
Nonpurified anti-IgM (method A)	Phosphate buffer	5.0, 5.3, 4.8	95.6, 94.6, 97.0
	Mouse serum	1.1, 1.3	93.8, 96.0
Purified anti-(kappa chain) (method A)	Phosphate buffer	36.8, 37.3, 37.1	97.3, 98.0, 96.5
Purified anti-IgM (method B)	Phosphate buffer	45.8, 43.2, 48.2	93.5, 96.7, 89.1
	Mouse serum	32.1, 27.5	87.5, 89.5
Nonpurified anti-IgM (method B)	Phosphate buffer	7.4, 7.8	
	Mouse serum	1.2, 1.3	

* Binding of IgM in the presence of 25, 5 and 1% (v/v) human serum was 2.7, 10.2 and 18.0% of added.

Table 3. *Binding in vivo of IgM to liposomes bearing anti-IgM IgG*

Mice were injected intravenously with 0.2 ml of phosphate buffer containing 60 μg of ^{125}I -labelled IgM [(2–5) $\times 10^5$ c.p.m.] and 3 min later with 0.2 ml of liposomes (1.8 mg of egg phosphatidylcholine) containing 0.2 M-carboxyfluorescein and coated with anti-IgM IgG (0.06–0.10 mg) or non-purified IgG (0.05 mg) by method A or B. Animals were killed 5 min after the last injection, blood serum was assayed for carboxyfluorescein latency and then chromatographed to separate free from liposome-bound IgM. Bound IgM is expressed as % ($\pm$ s.d.) of injected dose per total mouse serum (for details see the Materials and methods section). The numbers in parentheses denote the number of animals used.

IgG in liposomes	IgM bound (%)	Liposomal stability in blood serum (% latent carboxyfluorescein)
Purified anti-IgM (method A)	36.1 ± 2.0 (5)	83.0 ± 3.2 (5)
Non-purified (method A)	0.0 (5)	96.6 ± 2.8 (5)
Purified anti-IgM (method B)	42.8, 37.7 (2)	80.5, 82.3 (2)
Non-purified (method B)	0.0, 0.0 (2)	97.8, 95.5 (2)

Fig. 2. Separation of liposome-bound and non-reacted IgM

^{125}I -labelled IgM and carboxyfluorescein-containing liposomes bearing ^{131}I -labelled anti-IgM IgG were incubated separately (a) or together (b) in mouse serum at 37°C and then passed through Sepharose CL 6B. In a typical interaction experiment shown here (b), carboxyfluorescein (dotted line) and ^{131}I -labelled IgG (broken line) associated with liposomes are eluted together with the portion of liposome-bound ^{125}I -labelled IgM (solid line). Unreacted IgM and the small portion of liberated carboxyfluorescein are eluted later. The integrity of liposomes (fractions 8–13) is shown by the high (>98%) carboxyfluorescein latency. There was no ^{125}I or ^{131}I radioactivity in fractions 8–13 when appropriate amounts of ^{125}I -labelled IgM and free ^{131}I -labelled IgG were incubated together in serum. Similar elution profiles are obtained when interaction of liposomes and IgM occurs *in vivo* (see the Materials and methods section). In control experiments *in vivo*, after injection of either liposomes or ^{125}I -labelled IgM, liposomal markers and ^{125}I radioactivity are eluted as in (a). Again, there was no radioactivity in fractions 8–13 when appropriate amounts of ^{125}I -labelled IgM and free ^{131}I -labelled IgG were injected successively. Recoveries of chromatographed radioactivity and carboxyfluorescein in all experiments *in vitro* and *in vivo* were 92–104% of the quantities applied to the columns.

anti-IgG IgG onto liposomes provides a stock of stable liposomes which can easily be coated with the IgG required for targeting (method B). This allows for total availability of the targeting IgG to the environment (Table 1), in turn ensuring greater efficiency of interaction with the antigen, in this case IgM (Table 2).

It now remains to be seen whether our findings of targeting *in vivo* can be applied to other circulating

molecules such as drugs, hormones, etc. or to antigens associated with cellular targets. However, successful application of targeted liposomes *in vivo* is expected to require control over their rate of clearance in the blood. This should, in turn, influence the chances of contact between liposomes and target. Recent work (Gregoriadis & Senior, 1980) has shown that such control can be effected by the correct choice of the liposomal phospholipid component. For instance, in the case of small unilamellar liposomes composed of sphingomyelin their half life in the blood of mice was extended to 16 h (Gregoriadis & Senior, 1980; Hwang *et al.*, 1980).

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