Antibody-coated liposomes

The role of non-specific antibody adsorption

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Incubation of small unilamellar liposomes composed of equimolar phospholipid and cholesterol with mouse IgG or mouse monoclonal IgG_1 for up to 24 h resulted in considerable (34–89%) adsorption of the protein onto the liposomal surface. Immunoglobulin remained adsorbed after exposure to mouse plasma and, in the case of monoclonal (anti-HBSAg) IgG_1, was able to mediate association of the liposomes with the antigen. Extensive immunoglobulin adsorption suggests caution in interpreting covalent linkage values obtained upon prolonged incubation of chemically modified antibodies with liposomes. It may also be a preferred alternative in preparring targeted liposomes for intravenous use when chemically modified antibodies promote premature clearance of the antibody-liposome complex from the circulation.

Liposome *Drug targeting* *Antibody* *Monoclonal anti-HBSAg IgG_1* *Liposomal stability*

1. INTRODUCTION

Coating of liposomes with ligands (e.g. antibodies and other cell-specific proteins) which can mediate the uptake of the carrier by cells bearing the appropriate receptors is of growing importance to those interested in drug targeting [1,2]. A number of methods [1–3] have been developed for the association of protein ligands with the liposomal surface by covalent coupling and a particularly popular one employs the heterobifunctional *N*-hydroxysuccinimidyl-3-(2-pyridyldithio)-propionate (SPDP) [4,5] as the coupling reagent. It consists of modifying the protein to the 3-(2-pyridyldithio)propionate (PDP) derivative (protein-PDP), subsequently reduced to protein-SH with dithiothreitol (DTT). Similarly, phosphatidylethanolamine (PE) is modified with SPDP to give PE-PDP and is then incorporated into liposomes. For small unilamellar liposomes, coupling occurs when liposomal PE-PDP and protein-SH are incubated for 24 h at room temperature [4].

In the course of recent work with small unilamellar liposomes coated with antibodies through the SPDP reaction, we observed that high 'coupling' values were obtained in control experiments in which SPDP-modified or non-modified liposomes were incubated with intact IgG immunoglobulin. Here we report experiments suggesting that coupling values obtained by the use of coupling methods entailing lengthy incubation periods may be due to some extent to passive adsorption of the immunoglobulin onto liposomes. Passively adsorbed antibodies are retained by liposomes upon prolonged exposure to blood plasma and capable of binding to respective antigens. This simple and mild procedure of liposome coating with antibodies may be preferable to techniques in which modification of immunoglobulins with coupling reagents promotes rapid clearance of the injected liposome-immunoglobulin complex from the circulation.

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2. MATERIALS AND METHODS

The sources and grades of egg phosphatidylcholine (PC) [6], [^{3}H]PC [6], dimyristoylphosphatidylcholine (DMPC) [6], dipalmitoylphosphatidylcholine (DPPC) [6], dipalmitoylphosphatidylethanolamine (DPPE) [3], distearoylphosphatidylcholine (DSPC) [3], SPDP [3] and carboxyfluorescein (CF) [3] were as previously reported. Immunoglobulins used included mouse IgG (Sigma), and the mouse monoclonal IgG_1 specific for the hepatitis B surface antigen (HBSAg) prepared as described [7]. ^{125}I-labelled tracer IgG was prepared by the method of Fratzer and Speck [8] (spec. act. 5×10^4–2.6×10^5 cpm per μg protein), mixed with unlabelled IgG and, when appropriate, interacted with SPDP to give protein-PDP [3,4]. The latter was reduced to protein-SH with DTT to reveal the PDP to protein ratio [4]. Beads coated with HBSAg were prepared by the method of Goodall et al. [7].

2.1. *Preparation of liposomes*

Small unilamellar liposomes composed of equimolar total phospholipid and cholesterol and containing 0.2 M CF were prepared [9,10] by sonication, followed by centrifugation at 100000 × *g* and passage of the supernatant containing the small liposomes through a Sepharose 6B column [10]. Average size (nm ± SD) of liposomes was 64.0 ± 45.9 with more than 80% of the vesicles in the range of 44–80 nm diameter [11]. Multilamellar [12] and large unilamellar or oligolamellar liposomes (reverse-phase evaporation vesicles) [13] were prepared as described without extrusion through polycarbonate membranes. Some of the liposomal preparations were made in the presence of DPPE-PDP (DPPE-PDP liposomes) which was prepared and its identity and purity tested as described in [4].

2.2. *Coating of liposomes with immunoglobulins*

CF-containing liposomes were coated with the appropriate ^{125}I-labelled immunoglobulin by the SPDP method [4]. The procedure includes a 24 h incubation of DPPE-PDP liposomes with the protein-SH at 22°C under gentle stirring [5] to avoid precipitation of the former. In control experiments, non-modified or DPPE-PDP liposomes were mixed with intact immunoglobulin and subjected to the same conditions employed in the SPDP reaction. In some experiments non-modified liposomes were incubated with increasing amounts of immunoglobulin and in others, various time periods of incubation were used. Separation of liposomes from added reagents at various stages of the procedure was carried out either by molecular sieve chromatography using a Sepharose 6B column [10] (small liposomes) or by centrifugation at 8000 × *g* for 30 min (large liposomes). As observed before [3], coupling did not induce CF leakage. Incorporation of the antibody onto liposomes was estimated on the basis of ^{125}I radioactivity recovered with the liposomal fraction (chromatography) or washed liposomal pellet (centrifugation). In some experiments, IgG was incubated on its own under similar conditions and then chromatographed or centrifuged.

2.3. *Incubation of liposomes with plasma*

On the day of their use, immunoglobulin-coated liposomes containing CF were chromatographed (Sepharose 6B) and then incubated with 0.1 M sodium phosphate buffer containing 0.8% NaCl and 0.02% KCl, pH 7.4 (PBS) or fresh mouse plasma at 37°C for 16 h. Subsequently the mixtures were tested [10] for CF latency (which reflects liposomal stability in terms of CF retention) and passed through a Sepharose 6B column to determine immunoglobulin retention by liposomes.

2.4. *Interaction of antibody-bearing liposomes with antigen-coated beads*

Liposomes bearing anti-HBSAg mouse monoclonal IgG_1 were incubated with beads coated with HBSAg for 16 h. Binding of liposomes to the antigen was determined by washing the beads with PBS and assaying these for ^{125}I radioactivity [3] and/or CF, the latter in the presence of Triton X-100 (5% final concentration).

3. RESULTS AND DISCUSSION

Interaction of SH-activated mouse IgG or mouse monoclonal anti-HBSAg IgG_1 with CF-containing PE-PDP liposomes after a 24 h incubation results in 18.6–52.0% of the proteins being associated with liposomes (not shown). Comparable values (5–45%) have been obtained by others [4] using similar (mouse immunoglobulin)

protein to lipid ratios. In our hands, however, high values (21–89%) corresponding to a maximum of 20 μg immunoglobulin per μmol phospholipid were also observed in experiments in which intact or SPDP-modified liposomes were incubated with intact IgG (table 1, 24 h). Although IgG adsorption values varied considerably there was no consistent trend when increasing amounts of IgG were used. Further, values did not appear to depend on liposomal phospholipid composition or on whether the phospholipid (DSPC) was SPDP-modified and were similar for IgG of two different sources (table 1). In addition, the possibility that IgG coeluted with liposomes in a precipitate form induced by stirring was eliminated: IgG stirred on its own, eluted with fractions expected to contain IgG. Table 1 also shows that adsorption of IgG was not rapid (less than 2% at 15 min). However, at 5 h there was already considerable adsorption, at least for 3 of the preparations used. IgG adsorption by multilamellar or large unilamellar or oligolamellar liposomes made of lipid compositions similar to those employed for small liposomes and exposed to the protein under identical conditions, was much less extensive (0.7–2.9% of the protein used, table 1 legend).

Results (table 1) with small unilamellar liposomes are not in agreement with those obtained by Barbet et al. [4] who, in a similar control experiment with small liposomes, found very little antibody adsorption. However, their (mouse) antibody was of the IgG_{2a} type [4] and this difference (in the present work the Ig was either total IgG fraction from mouse plasma or mouse monoclonal IgG_1) may have contributed to IgG adsorption in our work. Indeed, Weissmann et al. [14], who studied the liposomal membrane-perturbing activity of certain Ig, have found that whereas IgG_1 is highly active in this respect, IgG_2 is inert.

Although IgG adsorption on prolonged incubation cannot be obviously related to the liquid-crystalline phase transition temperature of the phospholipids used (table 1), its extensive occurrence with small but not with large liposomes may

Table 1

Immunoglobulin adsorption onto small unilamellar liposomes

Liposomes	% of immunoglobulin added		
	15 min	5 h	24 h
PC, CHOL	1.2	3.4	52.0, 50.5*
DMPC, CHOL	1.7	30.4	59.2
DPPC, CHOL	0.8	17.5	89.5
DSPC, CHOL	1.6	44.3	89.0, 50.3*, 44.0*
DSPC, DPPE[a], CHOL			43.2*, 50.1*
DSPC, DPPE[a]-PDP, CHOL			34.7 ± 11.6 (5)

[a] 4% of total phospholipid

Small unilamellar liposomes (0.8–2.6 μmol phospholipid) composed of equimolar total phospholipid and cholesterol and containing 0.2 M CF were incubated with 1–88 μg ^{125}I-labelled mouse IgG or mouse monoclonal IgG_1 (10^5–2×10^6 cpm) at 22°C. At time intervals, samples were tested for CF latency and chromatographed. Radioactivity measured in the pooled fractions containing liposomes (as judged by the presence of latent CF) is expressed as % (± SD when appropriate) of added mouse IgG or mouse monoclonal IgG_1 (indicated by an asterisk). Large multilamellar, unilamellar or oligolamellar CF-containing liposomes of compositions and phospholipid content as for small liposomes and incubated with 2–140 μg mouse IgG or mouse monoclonal IgG_1 adsorbed after 24 h 0.7–2.9% of the protein added. There was no significant loss of liposomal stability (all preparations) in terms of CF retention [10] upon incubation in the presence of immunoglobulin for 24 h with CF latency values being more than 94% of those observed prior to incubation

be explained on the basis of the stressed curvature of the former. This would encourage partial insertion of IgG into the lipid domain. Once within such domains, availability of hydrophobic regions of IgG may be promoted as a result of direct contact with the lipid acyl chains [15], thus leading to hydrophobic bonding between the protein and vesicles.

Regardless of the nature of the bond between IgG and liposomes, its stability in the presence of mouse blood plasma is considerable. For instance, after 16 h incubation of immunoglobulin-coated liposomes (4 different phospholipid compositions) at 37°C, most (72–92%) of the protein remained adsorbed and, for 3 of the preparations used, values were similar to those obtained in buffer (table 2). As indicated from CF latency values (not shown) which reflect CF leakage, the presence of immunoglobulin on liposomes did not increase bilayer permeability to the solute above levels observed upon incubation in buffer or levels previously [3,6,9,10] measured under similar conditions in liposomes devoid of immunoglobulin. Further, adsorbed immunoglobulin (anti-HBSAg monoclonal IgG_1) appeared to retain its antigen-binding properties as judged by IgG_1 radioactivity or CF measurements (table 3) of the antigen-coated beads previously exposed to liposomes bearing the immunoglobulin. This is compatible with previous findings [16] of Fab region availability of adsorbed IgG on the liposomal surface and has been predicted [17] on the basis of the existence of a 'hinge' region, located between the

Table 2

Retention of liposomal immunoglobulin in the presence of blood plasma

Liposomes	% immunoglobulin retained	
	PBS	Plasma
PC, CHOL	87.6	72.1
DMPC, CHOL	91.6	91.9
DPPC, CHOL	88.8	89.1
DSPC, CHOL	90.3	87.9

CF-containing small unilamellar liposomes composed of equimolar phospholipid and cholesterol (1.0–1.5 μmol phospholipid) and bearing adsorbed mouse ^{125}I-labelled IgG (0.2–0.8 μg protein; 3.2×10^4–2.5×10^5 cpm) were assayed for immunoglobulin retention following incubation in the presence of PBS or fresh mouse plasma (50% final concentration) at 37°C for 16 h. Liposomal permeability to entrapped solutes (as judged from CF latency values) after incubation was tested [10] and found similar to that obtained [6,9,10] with liposomes devoid of immunoglobulin

Table 3

Binding of liposomes bearing adsorbed anti-HBSAg antibody to HBSAg-coated beads

Liposomes	Adsorbed ^{125}I-labelled antibody (μg/mg phospholipid)	Bound to beads	
		Radioactivity (cpm)	Carboxyfluorescein (units)
PC, CHOL	0.00	nil, nil	N.D.
	0.02	5010, 3359	N.D.
	0.27	9218, 9220	N.D.
DSPC, CHOL	0.00	nil, nil	50, 37
	0.02	1782, 1642	1230, 780
	0.26	6738, 5625	1590, 1680

CF-containing small unilamellar liposomes (4 μmol phospholipid) composed of equimolar phospholipid and cholesterol with or without adsorbed monoclonal anti-HBSAg ^{125}I-labelled IgG_1 were incubated overnight with HBSAg-coated beads. Subsequently, beads were washed with PBS and assayed for ^{125}I radioactivity and CF (see section 2). Results shown are from two separate experiments

Fab and Fc portions of the IgG molecule [15]. This region, because of its high content in hydrophobic amino acids, probably has a U-shaped conformation [18] which is expected [17] to promote the incorporation of the hydrophobic hinge region into the liposomal bilayer thus externally exposing the Fab and Fc portions [15].

Extensive passive adsorption of immunoglobulins to small liposomes after prolonged incubation, without loss of vesicle stability (as judged by CF latency retentions, table 1 legend), raises the possibility of employing this simple procedure as a means of grafting antibodies onto the liposomal surface through bonds which appear reasonably stable in the presence of blood. This procedure would certainly be a preferred alternative to the one based on passive antibody adsorption onto liposomes but involving extensive sonication [16,19] which can be detrimental [19] to the protein. Under certain conditions it may also be preferable to coupling methods: structural modifications of antibodies by reagents used in such methods have not been ascertained properly to date in terms of antibody fate in vivo, especially when prolonged presence of antibody-coated liposomes in the circulation is required: although modified antibodies are known [1–5] to maintain their affinity for the relevant antigens and to mediate the uptake of the liposome moiety by target cells in vitro, they could bring about rapid removal of the associated liposomes by the reticuloendothelial system after intravenous injection. It remains to be seen whether passive antibody adsorption diminishes the long circulation time [6,9] of some of the types of small liposomes used in the present study.

REFERENCES

[1] Gregoriadis, G. (1984) Liposome Technology, vol.3, CPC Press, Boca Raton, FL.
[2] Toonen, P.A.H.M. and Crommelin, D.J.A. (1983) Pharm. Weekbl., Sci. Ed. 5, 269–280.
[3] Wolff, B. and Gregoriadis, G. (1984) Biochim. Biophys. Acta 802, 259–273.
[4] Barbet, J., Machy, P. and Leserman, L.D. (1981) J. Supramol. Struct. Cell Biochem. 16, 243–258.
[5] Martin, F.J., Hubbell, W.L. and Papahadjopoulos, D. (1981) Biochemistry 20, 4229–4238.
[6] Gregoriadis, G. and Senior, J. (1980) FEBS Lett. 119, 43–46.
[7] Goodall, A.H., Meek, F.L., Waters, J.A., Miescher, G.G., Janossy, G. and Thomas, H.C. (1982) J. Immunol. Methods 52, 167–174.
[8] Fratzer, P.J. and Speck, J.C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857.
[9] Senior, J. and Gregoriadis, G. (1982) FEBS Lett. 145, 109–114.
[10] Senior, J. and Gregoriadis, G. (1982) Life Sci. 30, 2123–2136.
[11] Senior, J., Gregoriadis, G. and Mitropoulos, K.A. (1983) Biochim. Biophys. Acta 760, 111–118.
[12] Gregoriadis, G. and Ryman, B.E. (1972) Biochem. J. 129, 123–133.
[13] Szoka, F. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. USA 75, 4194–4198.
[14] Weissmann, G., Brand, A. and Franklin, E.C. (1974) J. Clin. Invest. 53, 536–543.
[15] Vandenbranden, M., De Coen, J.L., Jeener, R., Kanarek, L. and Ruyschaert, J.M. (1981) Mol. Immunol. 18, 621–631.
[16] Gregoriadis, G., Meehan, A. and Mah, M.M. (1981) Biochem. J. 200, 203–210.
[17] Burgess, A.W. and Sheraga, H.A. (1975) Proc. Natl. Acad. Sci. USA 72, 1221–1225.
[18] Dorrington, K.J. and Tanford, C. (1970) Adv. Immunol. 12, 333–381.
[19] Huang, L. and Kennel, S.J. (1979) Biochemistry 18, 1702–1707.