The Preparation of Liposomes Bearing Human (HLA) Transplantation Antigens

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The incorporation of HLA antigens into liposomes is described. At and above the lipid-phase-transition temperature, between 50 and 80% of the added antigenic activity may be incorporated into liposomes in the form of multilamellar vesicles. The antigen appears to be asymmetrically orientated with respect to the lipid bilayer.

The purpose of this paper is to report the preparation of liposomes bearing human transplantation antigens (HLA-A and HLA-B antigens). Liposomes carrying such purified gene products of the major histocompatibility complex will be useful in at least three areas: firstly, in investigating the ability of liposomes bearing these different gene products to stimulate an immune response *in vitro* and *in vivo*; secondly, in examining the ability of such liposomes to function as targets for immune lysis mediated by antibody and complement, by antibody-dependent effector cells and by cytotoxic thymus-derived lymphocytes; thirdly, in attempting to characterize the lipophilic portion of these membrane-bound gene products, which have been shown to penetrate the lipid bilayer (Walsh & Crumpton, 1977).

HLA antigens are membrane glycoproteins, consisting of two subunits: a glycoprotein of mol.wt. 45000 in the native form (Springer *et al.*, 1974), which is non-covalently linked to a protein of mol.wt. 11600, identified as B2-microglobulin (Grey *et al.*, 1973). The antigens may be solubilized by limited proteolysis with papain (Sanderson & Batchelor, 1968), or by the action of ionic (Snary *et al.*, 1974) or non-ionic detergents (Springer *et al.*, 1974). Detergent solubilization is a prerequisite for the preparation of antigen in a form suitable for incorporation into synthetic lipid vesicles, because the lipophilic portion of the molecule is preserved intact. Proteolysed water-soluble antigen has lost this fragment (Springer & Strominger, 1976) and would therefore not be expected to integrate into the lipid bilayer. Although water-soluble antigen could be entrapped non-specifically in the aqueous phase of liposomes, this was found, as might be expected, to be of low efficiency, presumably because of the very small entrapped volume compared with the total

volume of the aqueous phase. In view of its small micelle size and protein-binding characteristics, the ionic bile salt sodium cholate was used as the detergent in this study. Most preparations of detergent-solubilized antigen have utilized cultured lymphocytes as a source of material (Springer *et al.*, 1974; Snary *et al.*, 1974), but this work utilizes antigen prepared from human spleen.

Materials and Methods

Lens culinaris haemagglutinin, prepared by the method of Howard *et al.* (1971), and *Ricinus communis* haemagglutinin (Miles Laboratories, Slough, Bucks., U.K.) were coupled to Sepharose 4B [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] by the CNBr technique (March *et al.*, 1974). Papain (EC 3.4.22.2), sodium cholate, Tween 40 (polyoxyethylene sorbitan monopalmitate) and synthetic phosphatidylcholines were obtained from Sigma. Trasylol, a polypeptide inhibitor of serine proteinases, was obtained from Bayer Pharmaceuticals (Haywards Heath, W. Sussex, U.K.).

Protein was measured by the technique of Lowry *et al.* (1951), with bovine serum albumin as standard, and phospholipid by the method of Lowry *et al.*, (1954). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by the method of Laemmli (1970).

Preparation of HLA antigen from human spleen

HLA antigens were assayed by their ability to inhibit the cytolysis of ⁵¹Cr-labelled lymphocytes, mediated by HLA alloantisera and complement (Sanderson, 1964). All operations during the preparation of antigen were performed at 4°C in the presence of 100 kallikrein inhibitor units of Trasylol/ml and 5mm-dithiothreitol. Membranes were prepared from homogenates of human cadaver spleens by extraction with 2% Tween 40 as described by Morris *et al.* (1975). Antigen was solubilized from membranes by 1%

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sodium cholate in 10mM-Tris/maleate buffer, pH7.5, 150mM-NaCl, 5mM-dithiothreitol and 100 kallikrein units of Trasylol/ml at a concentration of 10mg of protein/ml. After 1 h at 4°C, insoluble material was removed by centrifuging at 105000g for 1h. Glycoproteins were isolated by affinity chromatography on a column of *Lens culinaris* haemagglutinin-Sepharose 4B as described by Snary *et al.* (1974) and were applied to a column (2.25cm×90cm) of Sephadex G-200 equilibrated in the above buffer solution. Fractions containing HLA antigen activity were pooled and applied to a column of *Ricinus communis* haemagglutinin-Sepharose 4B in the same buffer. HLA antigen did not bind to the column. Antigen-containing fractions were pooled, concentrated to a protein concentration of 2mg/ml and stored frozen at -20°C. There was no detectable loss of activity over a 6-month period.

Preparation of papain-treated antigen

HLA antigen (1 ml) partially purified as described was incubated at 37°C with 100 µl of papain (40 units; 1.6mg of protein) and 100 µl of 10mM-L-cysteine. After 30min, 100 µl of 13mM-iodoacetic acid was added, and, after cooling to 4°C, the mixture was applied to a 2ml column of *Lens culinaris* haemagglutinin-Sepharose 4B. The column was washed with 2 bed volumes of 0.01M-Tris/maleate (pH7.5)/0.5% (w/v) sodium cholate, before elution with 2% α-methyl-D-mannoside in the same buffer. The eluted glycoproteins were concentrated by vacuum dialysis to approx. 0.8mg/ml, and were stored frozen at -20°C.

Preparation of liposomes

Liposomes were prepared by a modification of the cholate dilution technique (Racker *et al.*, 1975). Suspensions of dimyristoyl phosphatidylcholine and dilauroyl phosphatidylcholine at concentrations of 40mg/ml and 4mg/ml in 10mM-Tris/maleate (pH7.5)/150mM-NaCl were prepared by sonication for 1–3 min with a Mullard sonicator of 4mm diameter (type SL81, 60W). Antigen (100 µg; 2mg/ml) in 10mM-Tris/maleate (pH7.5)/0.5% (w/v) sodium cholate was mixed with between 20 µg and 6400 µg of lipid. Where necessary, additional sodium cholate (10%, w/v) was added to effect complete solubilization of the lipids. After 30min, the mixture was diluted with 14ml of 10mM-Tris/maleate (pH7.5)/150mM-NaCl. After a further 1h incubation, tubes were centrifuged at 40000g for 16h. The liposome pellet was resuspended in 200 µl of the above buffer by vigorous vortex-mixing, or by sonicating for up to 2min. Mixing of antigen and lipid, and subsequent dilution and centrifugation, were all performed at the same temperature, namely at 2, 23 or 30°C.

Results and Discussion

Although HLA antigen prepared from human spleen as described showed greater than 600-fold purification over the crude homogenate, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed bands in addition to those of mol.wt. 45000 and 11600 corresponding to detergent-solubilized HLA antigen. It was estimated that the antigen was 20–25% pure. In the absence of Trasylol, much of the 45000-mol.wt. subunit was degraded to 34000.

Table 1 shows that, in the presence of dilauroyl phosphatidylcholine, up to 80% of the antigen could be recovered in the liposome pellet. No further antigen was exposed after dissolving the liposomes in 10% sodium cholate. To demonstrate that incorporation of antigen into liposomes was dependent on the hydrophobic character of the molecule, detergent-solubilized antigen was treated with papain. This treatment degrades the 45000-mol.wt. subunit to one of 34000, which is no longer capable of binding to detergents. Table 1 shows that, although the papain-treated antigen retained a high HLA-A2 antigen inhibition titre, less than 5% of this activity could be detected in association with phospholipid after

Table 1. Incorporation of HLA-A2 antigen into liposomes

Liposomes were prepared at 2°C as described in the text, by using 1 mg of dimyristoyl phosphatidylcholine and 100 µg of detergent-solubilized or papain-treated detergent-solubilized HLA-A2 histocompatibility antigen in 0.5% sodium cholate. Liposomes were collected by centrifugation and tested in the ⁵¹Cr-release assay for HLA-A2 antigen content. Liposomes prepared in the absence of antigen were also tested for inhibition of ⁵¹Cr release, as was antigen diluted and centrifuged in the absence of lipid. Recovery of HLA-A2 antigen activity is expressed relative to the amount of detergent-solubilized antigen (A) or papain-treated detergent-solubilized antigen (F) used in the preparation of liposomes. Results are expressed as means ± 1 s.d. for the numbers of observations given in parentheses. No standard deviations are shown where no antigen was detected.

	Percentage recovery of HLA-A2 antigen
(A) Detergent-solubilized antigen	100
(B) Liposomes prepared in the presence of (A)	80 ± 25 (7)
(C) (B) retested after being dissolved in 10% sodium cholate	84 ± 23 (3)
(D) Liposomes prepared in the absence of (A)	<1 (3)
(E) (A) sedimented in the absence of lipid	<1 (3)
(F) Papain-treated antigen	100
(G) Liposomes prepared in the presence of (F)	<6 (3)

liposome formation. This might well represent entrapped rather than membrane-incorporated antigen. Exploitation of this phenomenon could serve to expand the work of others (Allison & Gregoriadis, 1974) in which liposomes have been used as a vehicle for transport of antigens, resulting in augmented immunogenicity. Entrapment of antigen will only occur, of course, at the same concentration as that of the aqueous phase, whereas incorporation of detergent-soluble antigen into membrane may allow a concentration effect.

A possibility is that antigen may adsorb on the liposome surface. Although it cannot be ruled out, it appears unlikely for the following reasons. HLA antigens are known to possess only a single epitopic determinant (Sanderson & Welsh, 1974), which must be associated with the hydrophilic portion of the larger protein chain, since the papain-soluble molecule completely retains the ability to inhibit alloantisera specifically. It is also clear from our experiments that, if adsorption occurs, it does so essentially via the hydrophobic tail of the molecule, because the papain-treated antigen does not associate with liposomes (Table 1). Furthermore it seems unlikely that the hydrophobic tail will adsorb on the zwitterionic polar lipid head groups on the liposome surface in preference to interacting with the fatty acid side chains. Such a preference is substantiated by the temperature-dependence of antigen incorporation. The lipid-phase-transition temperature (T_m) would not affect the polar head group of the lipid (which is the same for both dimyristoyl phosphatidylcholine and dilauroyl phosphatidylcholine); it is a measure of the crystal packing of the fatty acid side chains. Hence the fact that antigen associates with dilauroyl phosphatidylcholine at both 2 and 23°C, and with dimyristoyl phosphatidylcholine at 23 but not at 2°C, probably means that interaction occurs with the fatty acid side chains, because they are the only variable between the two experiments. The fatty acid side chains, being buried in the lipid bilayer and

associated with the hydrophobic part of the HLA molecule, leaves the hydrophilic antigenic portion exposed externally to the aqueous environment. Although this may be thought of as an adsorption between two hydrophobic molecules, this term is not usually reserved for such phenomena, but is taken to refer to purely surface interactions. We prefer the expression 'integration' to explain the phenomena described.

With coliphage M13 coat protein, insertion of the protein into liposomes formed by the cholera dilution technique occurs maximally at the lipid-phase-transition temperature (Wickner, 1976). This was not so for HLA antigens.

Table 2 shows the results of experiments in which HLA antigen was incorporated into dimyristoyl phosphatidylcholine or dilauroyl phosphatidylcholine vesicles at 2, 23 and 30°C. Incorporation into the latter (T_m 3°C) occurs equally well at all three temperatures, whereas the former (T_m 23°C) bind antigen at 23 and 30°C, but not at 2°C. Hence, although antigen was not incorporated into vesicles at temperatures below T_m , there was no evidence to suggest that incorporation was suboptimal above the T_m .

Fig. 1 illustrates the lipid requirement for optimal incorporation: 200 µg of lipid is sufficient to bind the maximum amount of antigen, equivalent to approx. 200 µmol of lipid/µmol of protein.

Electron microscopy (kindly performed by Mr. R. Pillinger of the Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent, U.K.) revealed that the liposomes were closed multilamellar vesicles of diameter between 0.1 µm and 0.2 µm. Taken in conjunction with the finding that detergent solubilization of liposomes did not lead to an increase in the inhibition titre, this suggests that antigen is asymmetrically orientated in the lipid bilayer. Such a finding is consistent with results obtained for the M13 coat protein (Wickner, 1976), in which unilamellar vesicles were generated, but it is

Table 2. *Effects of temperature on the incorporation of antigen into liposomes*

Antigen (100 µg; 50 µl) was incubated for 30 min at the temperature indicated in the presence of 1 mg of lipid. Subsequent dilution, centrifugation and resuspension of liposomes were performed at the same temperature. Liposomes were assayed for the recovery of organic phosphorus, protein and HLA antigens. Results are expressed as means ± 1 s.d. Most of the experiments were repeated at least twice, several of them were repeated three times and a few were repeated several times.

Temperature (°C)	Percentage recovery in liposomes					
	Dilauroyl phosphatidylcholine liposomes			Dimyristoyl phosphatidyl choline liposomes		
	Phosphate	Protein	HLA	Phosphate	Protein	HLA
2	71	55 ± 3	73 ± 17	100	7 ± 2	7 ± 2
23	79 ± 9	55 ± 5	67 ± 11	84 ± 9	45 ± 3	56 ± 6
30	83	50	49	89	65	85

Fig. 1. Incorporation of HLA-A2 antigen into liposomes as a function of added lipid

Liposomes were prepared at 2 (dilauroyl phosphatidylcholine) or 23°C (dimyristoyl phosphatidylcholine) in the presence of 100 μg of detergent-solubilized HLA-A2 antigen, and different amounts of lipid, as shown. Liposomes were tested for HLA-A2 antigen activity, and for protein. ●, HLA-A2 antigen incorporated into dilauroyl phosphatidylcholine liposomes. ■, Protein incorporated into dilauroyl phosphatidylcholine liposomes. ○, HLA-A2 antigen incorporated into dimyristoyl phosphatidylcholine liposomes. □, Protein incorporated into dimyristoyl phosphatidylcholine liposomes.

not easy to reconcile with the multilamellar nature of the liposomes generated in this study. One possible explanation is that liposomes are fully permeable to antibody, but this seems unlikely on two grounds: firstly, because of the appearance of closed vesicles in the electron microscope; secondly, because we have since demonstrated that a water-soluble enzyme, horseradish peroxidase, may be trapped inside antigen-bearing liposomes during preparation, yet with no apparent leakage over a 4-week period (M. J. Turner & A. R. Sanderson, unpublished work). Hence it appears likely that antigen is incorporated into the outermost bilayer only. Recently, evidence has been obtained that, by inclusion of a charged lipid, unilamellar vesicles may be obtained that also

demonstrate asymmetric orientation of the antigen (E. M. Willoughby, A. R. Sanderson & M. J. Turner, unpublished work).

Liposomes carrying histocompatibility antigens prepared in this way can be used in investigating the role of lipid in the immunogenicity of histocompatibility antigens, and in comparing different methods of immune lysis.

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