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Reconstitution of purified detergent-soluble HLA-A and HLA-B antigens into phospholipid vesicles

(membrane proteins/liposomes/detergent dialysis)

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ABSTRACT **Purified detergent-soluble human histocompatibility antigens (HLA-A and HLA-B) were reconstituted into phospholipid vesicles by mixing the protein and lipid together in the presence of either octylglucoside (octyl-β-D-glucopyranoside) or deoxycholate and removing the detergent by dialysis. The resulting preparation consisted of lipid vesicles containing all or most of the added protein. The protein in the vesicles was antigenically active, as demonstrated by specific binding to anti-β_2-microglobulin IgG-Sepharose beads and by specific inhibition of alloantibody and complement-mediated cytotoxicity. Protein incorporated into vesicles at a protein/phospholipid ratio of 1:10 showed an asymmetric distribution of the HLA-A and HLA-B molecules, with virtually all of the antigens oriented facing the external medium. Cleavage experiments with proteases showed that the molecule was attached to the vesicle membrane via the COOH terminus, consistent with its proposed structure in intact cellular plasma membranes. Electron micrographs of the vesicles showed 50–60 Å knobs on the outer surface similar to structures observed for other membrane proteins. HLA-A and HLA-B could also be incorporated into vesicles together with Semliki Forest virus membrane proteins. The resulting preparations should be useful in defining the molecular interactions involving HLA-A and HLA-B antigens in the immune response.**

The major histocompatibility complex is a genetic region that codes for several different cell surface antigens involved in graft rejection and the immune response (1). In particular, the highly polymorphic histocompatibility antigens H-2K and D in mice and HLA-A and HLA-B in humans are coded for by genes in this region. These molecules appear to be recognized as target antigens by cytotoxic T lymphocytes, either alone (2) or in association with other cell surface structures (3–5). It also seems likely that these antigens are involved in the initial differentiation of these cells in the course of an immune response. The evidence to support this hypothesis has been gained primarily from experiments using inbred strains of mice and is therefore based on genetic arguments, experiments with interacting populations of intact cells, and well-characterized antisera. However, determination of the precise molecular interactions involved in these recognition events must ultimately rely on the purification, reconstitution, and biochemical analysis of the cell surface components presumed to be involved.

Recent reports from this laboratory have detailed the purification of HLA-A and HLA-B antigens from human lymphocytes in a detergent-soluble form (6, 7). The molecule consists of a 44,000-dalton glycoprotein (p44) associated with a 12,000-dalton protein that has been shown to be β_2-microglobulin (p12) (8). A model has been presented which suggests that the major portion of p44 is outside the membrane associated with β_2-microglobulin; a small portion near the COOH terminus passes through the membrane and a small hydrophilic portion of the COOH-terminus extends into the cytoplasm (refs. 9 and 10; unpublished data‡).

In this report we show that the detergent-soluble molecule can be reconstituted into lipid bilayer vesicles by a detergent dialysis method. We present evidence supporting an orientation and immunological activity of the molecule in vesicles that are similar to those in the native membrane.

MATERIALS AND METHODS

Deoxycholate was obtained from Schwarz/Mann. Octyl-β-D-glucopyranoside (octylglucoside) was synthesized by the method of Noller and Rockwell (11). Egg lecithin was type V-E from Sigma. All other chemicals were the finest grade obtainable.

Detergent-soluble HLA, a mixture of the allospecificities A2 and B7, was isolated from JY cells by the method of Robb *et al.* (7), using either Nonidet P-40 or Brij 99:97 (2:1). The purified protein was exchanged into either deoxycholate or octylglucoside by binding to a DEAE-cellulose column equilibrated in 10 mM Tris, pH 8.0/0.2% Triton X-100 or Brij 99:97. The column was washed with 10 column volumes of 10 mM Tris containing either 25 mM octylglucoside or 0.1% deoxycholate and was eluted with the same buffer containing 0.5 M NaCl. In some experiments, radiolabeled HLA-A and HLA-B antigens were prepared as described (12) by using neuraminidase, galactose oxidase, and finally [^{3}H]borohydride to label glycan chains in the membranes. Purification and detergent exchange were then performed as outlined above. In other experiments, the easily reduced half-cystines of the COOH terminus of the molecule were labeled with iodo[^{3}H[acetic acid as described (13). JY membrane lipids were extracted by the method of Bligh and Dyer (14). Semliki Forest virus proteins and ^{32}P-labeled BHK-21 cell lipids were isolated as described (15).

For reconstitution, a chloroform/methanol 2:1 (vol/vol), solution of JY lipid (0.1–1 mg) alone or egg lecithin (100 μg) and octylglucoside (400 μg) and 100 μl of ^{32}P-labeled BHK lipid were added to glass tubes and evaporated under N_2. The residue was redissolved twice in diethyl ether and reevaporated. A detergent solution (1 ml of 0.5% deoxycholate/0.14 M NaCl or 300 μl of 25 mM octylglucoside/0.5 M NaCl) containing 100 μg of HLA antigen was added to the dry lipid and dispersed with vortexing. Dialysis was for 64 hr against three 1-liter changes of 10 mM Tris, pH 8.0/0.14 M NaCl for deoxycholate or for 36 hr against two 1-liter changes of 4 mM Tris, pH 7.4/3

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Abbreviation: octylglucoside, octyl-β-D-glucopyranoside.

‡ Nguyen, Tu, (1978) Undergraduate Thesis (Harvard University, Cambridge, MA.) and T. Nguyen, D. Snackelford, R. Robb, and J. L. Strominger.

mM $MgCl_2$/0.1 mM dithiothreitol/2 mM NaN_3 with 0.3 M sucrose only in the first dialysis for octylglucoside. More than 99% of the detergents were removed by dialysis, as determined by using [^{3}H]deoxycholate or [^{14}C]octylglucoside (15).

Three types of sucrose gradients were used. For flotation gradients, the sample was added to 400 μl of 40 or 50% (wt/wt) sucrose in order to give a final concentration of 35–40%. A 5-ml gradient (30–0%) was layered on top of this sample. Centrifugation was for 16 hr at 190,000 × *g* and 4°. Fractions (0.25 ml) were collected from the bottom. Isopycnic gradients [5–40% (wt/wt) sucrose] consisted of 30 μl of sample layered on a 5.5-ml gradient that was centrifuged and fractionated as above. Velocity centrifugations were performed in 3–15% sucrose gradients with a 60% sucrose cushion at the bottom. Centrifugation was for 190,000 × *g* for 2.5 or 16 hr. Sucrose concentrations were determined with a refractometer. In some cases, flotation in stepwise sucrose gradients was used preparatively to separate the reconstituted vesicles from unreconstituted protein. After reconstitution, solid sucrose was dissolved into the reconstitute to give a concentration of 50% (wt/wt). The solution was placed at the bottom of a SW 50 gradient tube, overlaid with 1.5 ml of 40% sucrose in 0.05 M Tris·HCl, pH 7.4/0.10 M NaCl, and the tube was filled with buffer. After 16 hr of centrifugation at 4° at 50,000 rpm, fractions were collected and 10-μl aliquots were used for radioactivity measurements. The fractions containing radioactivity were pooled and dialyzed against 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 6.5/0.14 NaCl and finally concentrated about 2-fold with Sephadex G-150.

For immunoprecipitation, rabbit anti-human β_2-microglobulin IgG was coupled to Sepharose 4B (7) and equilibrated with 0.05 M Tris, pH 7.4/0.1 M NaCl/0.1% bovine serum albumin. To 0.1 ml of a 50% slurry, 25 μl of the reconstituted vesicles was added containing 5–10 μg of reconstituted protein. The mixture incubated for 2 hr at 4° with mixing. Then a 25-μl sample was withdrawn from the suspension for radioactivity determination, the beads were spun down with a table-top centrifuge, and a 25-μl sample was taken from the supernatant. From the difference in radioactivity, the amounts of free and bound material could be calculated.

Inhibition of cytotoxicity was assayed as described (6), using anti-HLA-A2 antisera. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed according to Laemmli (16), and staining was as described by Swank and Munkres (17). Protein was determined by the method of Lowry *et al.* (18), with bovine serum albumin as a standard. Negative staining for electron microscopy was done as described (15).

RESULTS

Purified HLA-A and HLA-B antigens were reconstituted into liposomes composed of egg lecithin, egg lecithin and cholesterol, or a crude mixture of lipid extracted from JY cell membranes. Antigen and lipids in either deoxycholate or octylglucoside could be used for reconstitution without affecting the results. Fig. 1 shows isopycnic (5–40%) sucrose density gradient profiles of material reconstituted with equal weights of HLA-A and HLA-B antigens and egg lecithin. The distribution of phospholipid shows that more than 85% occurred in particles with a buoyant density higher (1.03–1.12 g/cm^3) (Fig. 1*B*) than that of phospholipid reconstituted without protein (<1.03 g/cm^3) (Fig. 1*A*). When reconstitution was performed at a 1:10 protein/phospholipid ratio and the resulting material was run on a 3–15% sucrose gradient, a less pronounced increase in density was observed, from 1.023 to 1.048 g/cm^3, as a consequence of adding protein to the system (Fig. 2).

Flotation in isopycnic (5–40%) sucrose gradients indicated

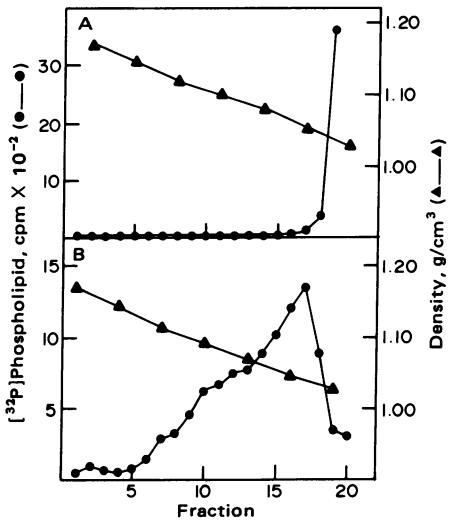

FIG. 1. Isopycnic (5–40%) sucrose density gradient profiles of lipid vesicles reconstituted from egg lecithin and octylglucoside. Reconstitution was carried out at an initial protein/phospholipid ratio of 1:1. (*A*) Vesicles in the absence of HLA-A and HLA-B antigens. (*B*) Vesicles containing HLA-A and HLA-B antigens.

that more than 80% of the HLA antigens reconstituted at a protein/phospholipid ratio of 1:1 were incorporated into phospholipid vesicles (Fig. 3). At a protein/phospholipid ratio of 1:10 all of the protein was incorporated into vesicles (Fig. 4*B*). In the absence of phospholipid, all of the protein was found at the bottom of the gradient (Fig. 4*A*).

The peaks of protein from sucrose density gradient profiles were calculated to have compositions of 125 μg of protein per mg of phospholipid and 500 μg of protein per mg of phospholipid for vesicles reconstituted at initial protein/phospholipid ratios of 1:10 and 1:1, respectively. In each case, the protein/phospholipid ratio within the peak increased with increasing density (Fig. 3 and unpublished data).

The immunological activity of the reconstituted vesicles, used to assay the vesicles in Fig. 4, indicated both that the antigens in the reconstituted vesicles were immunologically active and that at least a portion of the antigen was oriented with the alloantigenic site facing externally and accessible to antibody. That most of the vesicles thus obtained had some HLA-A and HLA-B antigens on their outer surface was also demonstrated by their binding to anti-β_2-microglobulin IgG coupled to

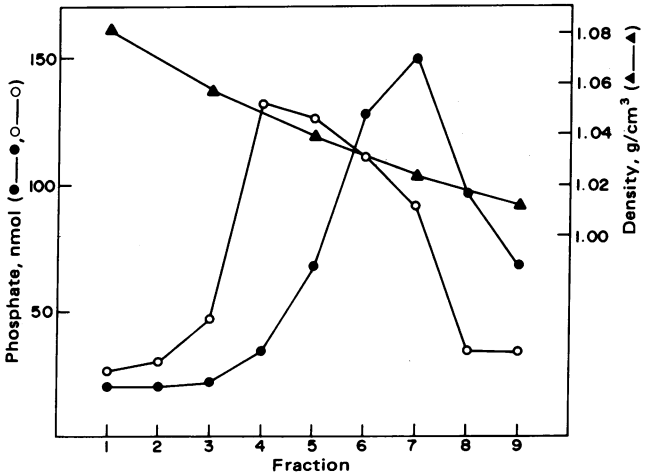

FIG. 2. Sucrose density gradient profiles of lipid vesicles reconstituted from JY lipid and deoxycholate in the presence (○) or absence (●) of HLA-A and HLA-B. Reconstitution was carried out at an initial ratio of 1:10 of protein to phospholipid.

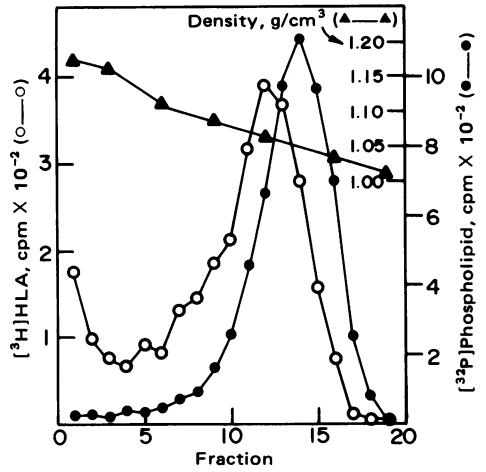

FIG. 3. Flotation of reconstituted [^{3}H]HLA-A and [^{3}H]HLA-B antigens and [^{32}P]phospholipid in an isopycnic (5–40%) sucrose gradient. Vesicles were made by using octylglucoside and egg lecithin, at an initial protein/phospholipid ratio of 1:1.

Sepharose 4B beads. Ninety percent of the [^{32}P]phospholipid was bound when the reconstituted vesicles were incubated with the beads; only 22% of similar vesicles reconstituted with Semliki Forest virus glycoprotein (15) was bound (Table 1).

In order to determine what percentage of the HLA antigenic activity was oriented externally, activity was measured before and after detergent solubilization (Table 2). Measurements were made on vesicles reconstituted in the presence and absence of 1 mM Ca^{+2}, which has previously been shown to cause aggregation of liposomes containing negatively charged phospholipids (19). Such aggregated liposomes can be pelleted by centrifugation whereas nonaggregated liposomes remain in the

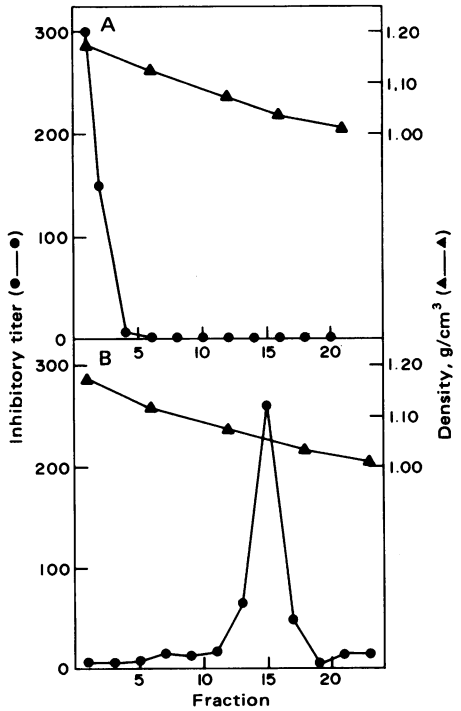

FIG. 4. Flotation of reconstituted HLA vesicles in an isopycnic (5–40%) sucrose density gradient. Vesicles were reconstituted by using JY lipid and deoxycholate at an initial protein/phospholipid ratio of 1:10. (*A*) HLA-A and HLA-B antigens dialyzed without phospholipid. (*B*) HLA-A and HLA-B antigens plus phospholipid. Inhibitory titer refers to the reciprocal of the dilution of the appropriate fraction giving 50% inhibition of cell lysis by alloantiserum and complement.

Table 1. Binding of reconstituted vesicles to anti-β_2-microglobulin IgG coupled to Sepharose beads

Vesicles reconstituted with	[^{32}P]Phospholipid bound, % of total	SFV [^{35}S] protein bound, % of total	Determinations, no.
HLA-A and HLA-B antigens	91		3
SFV spike proteins	22	27	1
HLA-A and HLA-B antigens plus SFV spike proteins	80	80	1

HLA-A and HLA-B antigens or ^{35}S-labeled Semliki Forest virus (SFV) spike proteins were reconstituted alone or together in [^{32}P]-phospholipid.

supernatant. More than 90% of the activity in liposomes made in the presence of 1 mM Ca^{+2} could be sedimented by centrifugation at 100,000 × *g* for 1 hr but less than 10% of the activity in those made in the absence of 1 mM Ca^{+2} could be sedimented during this time. A similar distribution of phospholipid phosphate was observed in the two types of reconstitution. On treatment with detergent the alloantigenic activity of the pelleted vesicles made in the presence of Ca^{+2} increased slightly, suggesting that a maximum of 30% of the total activity in these vesicles was not facing the external environment. In the absence of Ca^{+2}, only a small amount of activity was found in sedimentable liposomes and no significant change was observed on treatment with detergent.

When sedimentable liposomes were treated with papain and then resedimented, all of the alloantigenic activity was found in the supernatant and none in the pellet (Table 2). Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the material solubilized by papain showed it to consist of two polypeptide chains of molecular weights 34,000 and 12,000 (p-34,12) (data not shown). This corresponds to the form of HLA isolated by papain treatment of intact cells or cell membranes (20) and also to the products of papain treatment of purified detergent-soluble HLA (9).

In order to define more closely the nature of the attachment of HLA antigens to the membrane, a comparison was made of the susceptibility of the molecules to trypsin cleavage. Trypsin catalyzes a limited cleavage of p44,12 in the COOH-terminal

Table 2. Distribution of HLA alloantigenic activity in HLA vesicles

Treatment	Inhibitory titer $-Ca^{+2}$ liposomes	Inhibitory titer $+CA^{+2}$ liposomes
Centrifugation at 100,000 × *g*:		
Pellet	18	220
Supernatant	180	18
0.1% Brij added to 100,000 × *g* pellet	17	320
Papain treatment of 100,000 × *g* pellet and then recentrifugation:		
Pellet	0	20
Supernatant	25	230

Liposomes were prepared with JY lipid and deoxycholate at an initial protein/phospholipid ratio of 1:10 and dialyzed against buffer in the presence or absence of 1 mM $CaCl_2$. After dialysis, the samples were centrifuged at 100,000 × *g* for 1 hr, the supernate was removed, and the pellet was resuspended in the original sample volume. An aliquot of the pellet was treated with 0.1% Brij-containing buffer overnight at 4°C. Another aliquot was treated with papain (papain/protein ratio, 1:100) for 45 min at 37° and then centrifuged at 100,000 × *g* for 1 hr. The pellet and supernatant were assayed separately for activity. Liposomes reconstituted without HLA antigens had no cytotoxic inhibitory effect.

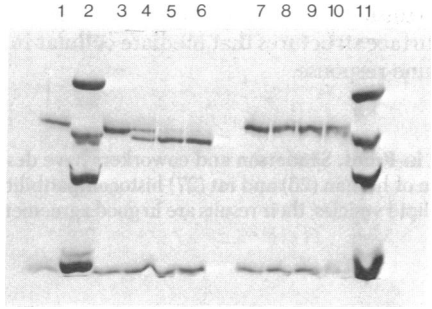

FIG. 5. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of HLA vesicles or HLA in 0.1% deoxycholate after treatment with trypsin. Lanes: 1, HLA in 0.1% deoxycholate (HLA-DOC); 2, protein standards; 3, HLA-DOC; 4, HLA-DOC plus trypsin (1:1,000); 5, HLA-DOC plus trypsin (1:200); 6, HLA-DOC plus trypsin (1:50); 7, HLA vesicles; 8, HLA vesicles plus trypsin (1:1,000); 9, HLA vesicles plus trypsin (1:200); 10, HLA plus trypsin (1:50); 11, protein standards. The protein standards are: bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (29,000), and lysozyme (14,000).

region to yield p39,12 (see *Discussion*). In the present experiment, addition of trypsin to detergent-soluble HLA resulted in complete cleavage of p44,12 to p39,12 (Fig. 5). In contrast, treatment of HLA-A and HLA-B antigens reconstituted in liposomes with trypsin did not result in any cleavage. This observation indicates that the COOH terminus of HLA-A and HLA-B antigens is rendered inaccessible to the external environment due to its association with the lipid vesicles.

Electron microscopy after negative staining made it possible to visualize the HLA molecules. Along the edges of the vesicles they were seen as spike-like projections 50–60 Å long and about 20–30 Å thick (Fig. 6). On the flat surface of the vesicles they appeared as light dots. The density of the dots was about 30/1000 nm^2. Vesicles reconstituted in the absence of proteins had a smooth surface.

When ^{35}S-labeled Semliki Forest virus spike protein (15), detergent-solubilized HLA-A and HLA-B antigens, and ^{32}P-labeled phospholipid were reconstituted together, vesicles were obtained that contained all three components. That the viral proteins had become lipid-bound was clear because more than 90% of the ^{35}S activity floated in a sucrose gradient together with the ^{32}P activity (data not shown). Eighty percent of both the ^{35}S and the ^{32}P activity in the floating material was bound to the anti-β_2-microglobulin IgG coupled to Sepharose, indicating that the vesicles also contained HLA molecules (Table 1). Electron microscopy showed that the material consisted of vesicles (average diameter, 1000 nm) with projections on their surface similar to those shown in Fig. 6. However, it was not possible to distinguish between HLA-A and HLA-B antigens and virus proteins in these vesicles because of their similarity in morphology.

DISCUSSION

Reconstitution of detergent-soluble HLA-A and HLA-B antigens with phospholipid resulted in association of the protein with phospholipid to form lipid–protein vesicles. The sucrose density gradient profiles showed that this association was dependent upon relative amounts of protein and lipid. At high protein/lipid ratios, it was possible to exceed the capacity of the lipid for protein, resulting in the formation of protein aggregates that could be visualized by electron microscopy (unpublished data). However, at lower protein/lipid ratios, essentially all of the protein was associated with lipid. Sucrose density gradient profiles indicated that only a single class of HLA vesicles was formed. There was no evidence of the formation of distinct

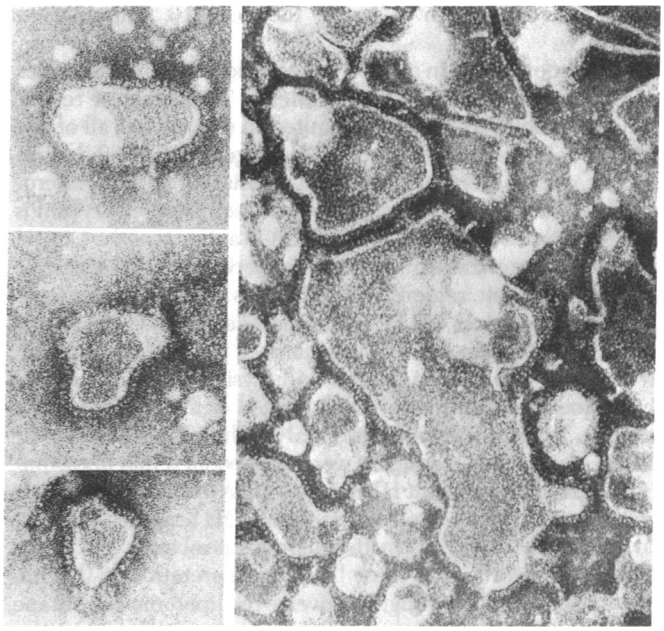

FIG. 6. Electron microscopy of negatively stained preparations of HLA vesicles. Reconstitution was from octylglucoside and egg lecithin at an initial protein/phospholipid ratio of 1:1. The sample was subsequently subjected to flotation in a stepwise sucrose density gradient and the phospholipid-containing fractions were used for analysis. (×290,000.)

subclasses of vesicles, as has been reported for reconstituted Semliki Forest virus spike proteins (15).

Electron microscopy showed the presence of a dense coat of knob-like structures protruding about 50–60 Å from the cell surface. It could be estimated that an average vesicle carried about 500–2000 HLA-A and HLA-B molecules on its surface. The morphology of the HLA-A and HLA-B antigens as shown by negative staining was very similar to that previously seen for the Semliki Forest virus spike proteins, although the latter extend somewhat further out from the membrane, consistent with their higher molecular weight. In the case of the Semliki Forest virus proteins, the length of the spikes seen after negative staining corresponds to that obtained by low-angle x-ray diffraction studies on the virus (15). This gives some confidence that the electron micrographic image may reflect the actual dimensions of HLA-A and HLA-B antigens also.

The reconstituted HLA-A and HLA-B antigens in vesicles were antigenically active as demonstrated by binding of the vesicles to anti-β_2-microglobulin IgG coupled to Sepharose and by their ability to inhibit the cytotoxicity of alloantigenic serum and complement. The immunological activity was increased only slightly when the liposomes were solubilized by detergent, indicating that most if not all of the molecules were asymmetrically oriented with the antigenically active region facing the outside of the liposome.

Lactoperoxidase-catalyzed iodination of HLA-A and HLA-B antigens in intact cells and on inside-out vesicles separated from the crude membrane fraction of these cells (10, unpublished data,‡) and isolated detergent-soluble HLA-A and HLA-B antigens (unpublished data‡), together with cleavage of the resulting labeled molecules by papain and CNBr (unpublished data‡), has shown that the HLA-A and HLA-B antigens are oriented with the NH_2-terminal 34,000-dalton piece on the outside of the cell associated with β_2-microglobulin. The external piece is linked to a small hydrophobic peptide of about 26 amino acids which traverses the membrane (R. Robb, personal communication) and the COOH-terminal hydrophilic

region (9, 21) which is inside the cell (10, unpublished data‡).

Further evidence in support of this orientation in liposomes was provided by protease experiments. Treatment of reconstituted vesicles with papain resulted in cleavage of all of the 44,000-dalton subunit of the HLA antigens to 34,000 daltons and its release from the vesicle in association with β_2-microglobulin, as occurs with intact cells or membranes. Trypsin is believed to cleave to HLA-A and HLA-B antigens at the COOH terminus, somewhere in the junction between the hydrophilic and hydrophobic regions where there is a cluster of arginine and lysine residues (22§). The remainder of the molecule is resistant to trypsin unless it is denatured (despite the fact that it contains about 50 Arginine and Lysine residues). As the consequence, trypsin catalyzes a limited cleavage of p44,12 to p39,12. Trypsin was unable to catalyze this cleavage when the HLA-A and HLA-B antigens were incorporated into liposomes. These data indicate that HLA-A and HLA-B antigens associate with the phospholipid bilayer via the COOH terminus, the same arrangement as occurs in the plasma membrane (9, 10, unpublished data‡). The most likely interpretation is that the HLA-A and HLA-B antigens traverse the liposome membrane, as they do that of the intact cell. However, without repetition of the lactoperoxidase-catalyzed iodination experiments, the data do not unambiguously exclude a hairpin structure in which the hydrophilic COOH-terminal region is on the outside surface of the liposome but associated with it in some manner that renders it inaccessible to trypsin. Thus, the results of this paper are consistent with the idea that all of the HLA antigens reconstituted in vesicles have an arrangement similar to that of the molecules in the native membrane. This arrangement appears to be independent of the lipid or detergent used for reconstitution. This contrasts with the reconstitution of Semliki Forest virus membranes (15) in which octylglucoside was clearly better than deoxycholate in giving active, nonaggregated vesicles.

This general approach for solubilization and reconstitution of membrane surface antigens should have wide application in many systems. As was demonstrated here, it is possible to reconstitute Semliki Forest viral membrane proteins and HLA-A and HLA-B antigens into the same vesicle. In another paper, these proteins in separate vesicles will be used to demonstrate that HLA-A and HLA-B antigens are the receptors on cell surface for Semliki Forest virus (22). In addition, p29,34 (a product of the *HLA-D* locus and the human analog of the murine Ia antigen) has been reconstituted into lipid vesicles in an antigenically active form (unpublished data). The use of reconstituted systems rather than solubilized material in exploring biological functions may be important in situations in which multivalent interactions between two membrane surfaces are crucial in eliciting appropriate responses.

There have been several reports that detergent-solubilized (23) and reconstituted (23, 24) plasma membranes from mice are capable of causing stimulation of allogeneic cell-mediated lympholysis reactions in mice *in vitro*. In addition, it has recently been reported that a preparation consisting of reconstituted H-2 and Ia molecules was capable of eliciting secondary allogenic mixed leukocyte culture responses *in vitro* (25). In preliminary experiments, liposomes containing HLA-A2 and HLA-B7 described here were found to elicit secondary stimulation of cytotoxic lymphocytes in a xenogeneic murine system (unpublished data). It should also be interesting to determine whether this material is similarly active in an allogeneic or modified syngeneic response *in vitro*. This approach should contribute to the definition, at the molecular level, of the nature of the cell surface structures that mediate cellular interactions in the immune response.

§ DeFranco, A. (1975) Undergraduate Thesis (Harvard University, Cambridge, MA.).

Note Added in Proof. Sanderson and coworkers have described the reconstitution of human (26) and rat (27) histocompatibility antigens into phospholipid vesicles; their results are in good agreement with our own.

Anti-β_2-microglobulin-Sepharose 4B was a generous gift of Dr. Richard Robb. This work was supported in part by Research Grant AI 10736 from the National Institutes of Health to J.L.S., by a short-term European Molecular Biology Organization Fellowship to C.T., and a U.S. Public Health Service Fellowship to V.H.E.

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