Self-Association of Class I Major Histocompatibility Complex Molecules in Liposome and Cell Surface Membranes†

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ABSTRACT: Fluorescent derivatives of a human MHC class I glycoprotein, HLA-A2, were reconstituted into dimyristoylphosphatidylcholine (DMPC) liposomes. Measurements of lateral diffusion of fluorescein- (Fl-) labeled HLA-A2 by fluorescence photobleaching recovery (FPR), of rotational diffusion of erythrosin- (Er-) labeled HLA-A2 by time-resolved phosphorescence anisotropy (TPA), and of molecular proximity by flow cytometric fluorescence resonance energy transfer (FCET) showed that these class I MHC molecules self-associate in liposome membranes, forming small aggregates even at low surface concentrations. The lateral diffusion coefficient (D_{lat}) of Fl-HLA-A2 decreases with increasing surface protein concentration over a range of lipid:protein molar ratios (L/P) between 8000:1 and 2000:1. The reduction in D_{lat} of HLA molecules in DMPC liposomes is found to be sensitive to time and temperature. The rotational correlation time for Er-HLA-A2 in DMPC liposomes at 30 °C is 87 ± 0.8 μs, at least 10 times larger than that expected for an HLA monomer. There is also significant quenching of donor (Fl-HLA) fluorescence at 37 °C in the presence of acceptor-labeled (sulforhodamine-labeled HLA) protein indicating proximity between HLA molecules even at L/P = 4000:1. FPR and FCET measurements with another membrane glycoprotein, glycophorin, give no evidence for its self-association. HLA aggregation measured by FPR, FCET, and TPA was blocked by β2-microglobulin, b2m, added to the liposomes. The aggregation of HLA-A2 molecules is not an artifact of their reconstitution into liposomes. HLA aggregates, defined by FCET, were readily detected on the surface of human lymphoblastoid (JY) cells. These cells are known to display some class I HLA molecules lacking b2m.

Major histocompatibility complex (MHC)[1] class I molecules are integral membrane proteins having 1–3 glycosylation sites and a single-span transmembrane domain. Their main function is in antigen presentation to T-cells. In addition there is physical and biochemical evidence that they can associate with other proteins, notably peptide hormone receptors, in the plane of the cell-surface membrane, perhaps influencing the functions of these proteins (Schreiber et al., 1984; Phillips et al., 1986; Szollosi et al., 1987, 1989; Blue et al., 1988; Edidin & Reiland, 1990; Liegler et al., 1991; Ferm & Gronberg, 1991; review, Edidin, 1988). There is also conflicting evidence that MHC class I molecules may self-associate at the surface (Cresswell & Dawson 1975; Snary et al., 1975; Damjanovich et al., 1983; Szollosi et al., 1989).

The mode of formation and the stoichiometry of complexes of surface MHC molecules has not been well defined. While class I MHC molecules associated with other surface receptors can usually be isolated with antibodies reacting against native molecules, and even with antibodies to the class I MHC light chain, β2-microglobulin (b2m) (e.g., Phillips et al., 1986), this is not always the case (Bushkin et al., 1988). The possibility remains open that free class I heavy chains play a role in initiating or stabilizing these associations.

Purified MHC molecules incorporated into synthetic phospholipid vesicles, liposomes, offer a simple system for the study of lateral complex formation by class I MHC molecules. Here we show that human MHC class I molecules, HLA-A2, aggregate when reconstituted into liposomes at low protein concentrations. Measurements of lateral diffusion, of rotational diffusion, and of molecular proximity all show that HLA-A2 molecules form aggregates with radii 4–5 times that of an HLA monomer. Aggregation depends upon protein concentration and temperature. The protein concentrations at which complexes form are much lower than those reported for aggregation of other proteins reconstituted into liposomes, and the effects seen for HLA molecules are not found for liposomes containing another purified membrane glycoprotein, glycophorin. Aggregates dissociate and their formation is blocked if exogenous b2m is added to liposomes.

Self-association of class I MHC molecules can also be detected on the surfaces of B-lymphoblasts which bear appreciable amounts of b2m-free class I molecules.

MATERIALS AND METHODS

Protein A–Sepharose, Sephadex G-25, G-50, cyanogen bromide-activated Sepharose 4B, deoxycholic acid (DOC), dimyristoylphosphatidylcholine (DMPC), soybean phosphatidylcholine (SL), glycophorin from human blood, type MM, and β2-microglobulin, b2m, from human urine were purchased from Sigma (St. Louis, MO). Octyl glucoside was from Calbiochem (La Jolla, CA). Fluorescein isothiocyanate (FITC), sulforhodaminesulfonyl chloride (Texas red), and erythrosin isothiocyanate (ErITC) were obtained from Molecular Probes (Junction City, OR). NBD-PC (1-palmitoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]phosphatidylcho-

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[1] Abbreviations: MHC, major histocompatibility complex; DMPC, dimyristoylphosphatidylcholine; SL, soybean lecithin; FITC, fluorescein isothiocyanate; Texas red, sulforhodaminesulfonyl chloride; ErITC, erythrosin isothiocyanate; PBS, phosphate-buffered saline; HLA, human leukocyte antigen; b2m, β2-microglobulin; FPR, fluorescence photobleaching and recovery; TPA, time-resolved phosphorescence anisotropy; FCET, flow cytometric energy transfer.

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line) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL).

Hybridoma cells (HB82, American Type Culture Collection, Rockville, MD), secreting mAb BB7.2 specific to HLA-A2 (Parham & Brodsky, 1981), were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories Inc., Grand Island, NY) supplemented with 10% fetal calf serum (Intergen, Purchase, NY). The mAb was purified by affinity chromatography on a protein A–Sepharose column (Ey et al., 1978). A sample of mAb reacting with b2m-free HLA heavy chains, HC10 (Stam et al., 1986), was kindly provided by Dr. Yuri Bushkin (Public Health Research Institute, New York).

CNBr-activated Sepharose was derivatized at a concentration of 2–3 mg of BB7.2/g of dry gel in a buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.4, overnight at 4 °C. The unreacted binding sites were blocked by resuspending the gel in 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.3.

Cells of the JY human B-lymphoblastoid cell line were cultured to a total of 10^{10} cells. A bulk culture of these cells was kindly grown for us by Dr. Joseph Shiloach, NIDDK, NIH. The 1% Triton X-100 extract of these cells was used for the purification of HLA-A2.

Purification of HLA-A2 from JY Cell Lysate. HLA-A2 molecules were purified by affinity chromatography on a BB7.2–Sepharose column as described by Parham (1983). The JY cell lysate was passed in series through (1) a Sepharose 4B precolumn, (2) Sepharose 4B coupled with normal mouse IgG, and (3) a BB7.2–Sepharose column. Column 3 was then washed extensively with PBS containing 0.5% Triton X-100 followed by 0.5% octyl glucoside in PBS. The bound antigens were eluted with 50 mM diethylamine containing 0.5% octyl glucoside and 0.15 M NaCl, pH 11.5. The eluted fractions were neutralized with 0.1 M Tris-HCl, 0.15 M NaCl, and 0.5% octyl glucoside, pH 6.9, and rechromatographed on a BB7.2–Sepharose column before reconstitution experiments.

The purity of protein was checked by SDS–polyacrylamide Gel electrophoresis (Laemmli, 1970). A single main band was observed at ~45 kDa, but traces of minor bands were also seen.

Preparation of Fluorescent Derivatives of Purified Membrane Proteins. Affinity-purified HLA-A2 was allowed to react with a 5-fold molar excess of FITC (Fl) or sulforhodaminesulfonyl chloride (SR) in a buffer, 0.1 M $NaHCO_3$ containing 0.5% octyl glucoside, pH 9.5, at 4 °C. After 16 h of reaction, the free dye was separated from the protein by gel filtration on a Sephadex G-50 column in 10 mM Tris-HCl, 100 mM NaCl, and 0.5% octyl glucoside, pH 7.4. The labeled protein was then repurified by affinity column chromatography as described above.

Glycophorin was labeled with FTIC or sulforhodaminesulfonyl chloride in 0.1 M sodium borate, pH 9.0, at 4 °C as described by Vaz et al. (1981). The free dye was separated from the protein by gel filtration on a Sephadex G-25 column.

The fluorescein/protein molar ratios of HLA-A2 and glycophorin were 1.18 and 2.35, respectively. The sulforhodamine/protein molar ratios of HLA-A2 and glycophorin were 1.94 and 2.60, respectively. The protein content was determined by the method of Lowry (1951). The fluorescein concentration was estimated from absorbance at 495 nm (molar absorptivity 63 000 M^{-1} cm^{-1}) and the SR concentration from absorbance at 594 nm (molar absorptivity 85 000 M^{-1} cm^{-1}). Gel electrophoresis of the fluorescently labeled HLA-A2 showed one major band corresponding to 45 kDa and a smear of weak fluorescence at lower apparent M_r.

Labeling of HLA-A2 with Erythrosin Isothiocyanate. HLA-A2 in 0.5% octyl glucoside was dialyzed extensively against 0.1 M $NaHCO_3$ and 0.1 M NaCl, pH 8.2, containing 0.5% DOC. The protein was then reacted with a 10-fold molar excess of ErITC dissolved in a small volume of dimethylformamide at 4 °C. After 18 h of reaction the free dye was separated from the protein by gel filtration and the labeled protein was repurified by affinity column chromatography. The erythrosin concentration was estimated from the absorbance at 535 nm (molar absorptivity 101 000 M^{-1} cm^{-1}) and the dye/protein molar ratio was 1.90.

Preparation of Giant Proteoliposomes. Giant proteoliposomes suitable for FPR measurements were made by a modification of the method of Keller et al. (1988). Protein in detergent was mixed with lipid and dialyzed against buffer to remove detergent. For FPR the small proteoliposomes were made into micrometer-diameter liposomes by the dehydration followed by rehydration technique. Octyl glucoside was used as the detergent (Mimms et al., 1981).

A thin film of DMPC was deposited on the wall of a glass tube from a solution in chloroform/methanol (2:1 v/v) by evaporation under dry nitrogen. The film was dried in a desiccator containing $CaCl_2$ for at least a day. The lipid film (2.7 μmol of DMPC) was then hydrated with a buffer containing 10 mM Tris-HCl and 100 mM NaCl, pH 7.4 (TSB), with an 8–10-fold molar excess of octyl glucoside. After the addition of fluorescently labeled proteins to the optically clear suspension of DMPC/octyl glucoside mixed micelles, the volume was adjusted to a final concentration of 30 mM octyl glucoside. The lipid/protein ratio was always varied by changing the amount of protein added. The mixture of lipid and protein in octyl glucoside was dialyzed against a 1000-fold volume excess of TSB for 5–6 h at 4 °C until vesicles formed and settled in the dialysis bag.

Calorimetric measurements showed that the lipid gel–liquid crystalline phase transition temperature of the dialyzed DMPC proteoliposomes was centered at 23.9 °C, indicating that the octyl glucoside was completely removed by the 5–6 h of dialysis and that the protein concentrations used did not significantly perturb the bilayer.

Once formed, vesicles were centrifuged out of solution at 15 000 rpm for 10 min at 4 °C. More than 90% of the total input of proteins was found to be associated with the pellet. The pellet was then resuspended in 20 μL of 10 mM MOPS buffer, pH 7.4, containing 100 mM NaCl and 5% (w/v) ethylene glycol. A portion of the suspension (10 μL) was deposited on a glass slide, avoiding excessive spreading. The drop of liposome suspension was dehydrated in a desiccator for about 2–3 h. The partially dehydrated film was then covered with 10–15 μL of distilled water and rehydrated for 2–4 h at room temperature to form giant proteoliposomes. These were then taken up in 50-μm rectangular section capillary tubes (Vitro Dynamics, Millville, NJ) and kept for 16 h at 4 °C. Before FPR measurements, liposomes were equilibrated to room temperature for 1 h.

The samples in the capillary tubes contained large spherical liposomes, with typical diameters of 20–50 μm, at the edges of jumbled masses of proteolipid (Figure 1). They showed an almost uniform fluorescence throughout and were assumed to be multilamellar. Some large, thin, apparently paucilamellar liposomes were also formed. No differences in the fluorescence recovery curves were observed between apparently paucilamellar and multilamellar liposomes.

Liposomes containing erythrosin-labeled HLA-A2 and sulforhodamine-labeled HLA-A2 were made by the dialysis method described for Fl-HLA liposomes. They were used for the measurement of rotational diffusion (Er-HLA) and for flow cytometric energy transfer measurements (SR-HLA) as

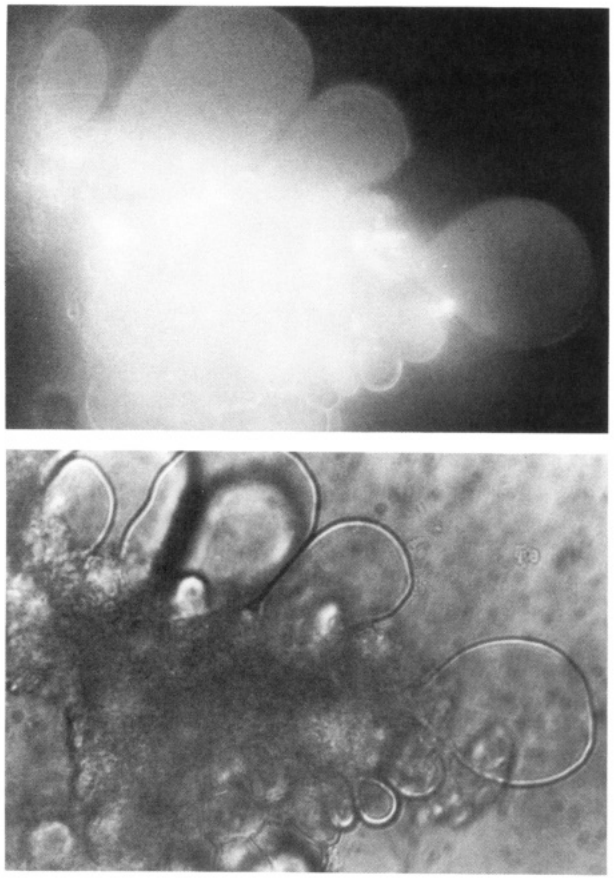

FIGURE 1: Large Fl-HLA-containing proteoliposomes formed after partial dehydration and rehydration of small DMPC proteoliposomes. (Top) Fluorescence. Faintly fluorescent paucilamellar vesicles are growing out of a jumbled lipid mass. (Bottom) Bright field.

described below. In these cases the liposomes obtained after detergent dialysis were centrifuged to a pellet and then the pellet was suspended in Tris–saline buffer (pH 7.4) for measurements.

Labeling with Antibody and Antibody Fragments. Fluorescein-Fab fragments of mAbs BB7.2 (anti-HLA-A2) and 28-14-8 (anti-H-2L^d) (Ozato et al., 1980) were used to label liposomes containing HLA-A2. Preparation and labeling of mouse Fab was according to our published methods (Edidin & Stroynowski, 1991).

The partially dehydrated lipid film, deposited on a glass slide, was treated with the Fl-Fab fragments at room temperature for 15 min in TSB. THe excess conjugates were then removed by touching the edge of a piece of filter paper to the solution. The film was washed 4–5 times with 20-μL batches of TSB.

Measurement of Lateral Diffusion. We used spot photobleaching (Wolf, 1989) to measure lateral mobility of fluorescently labeled membrane components. Briefly, an attenuated, focused laser beam was used to define a spot on the surface of liposomes. The spot was then bleached for 10–20 ms with the full-power laser beam. The beam was attenuated again and used to monitor the time-dependent recovery of the fluorescence in the bleached spot. Such experiments measure two parameters: D, a diffusion coefficient for mobile molecules, and R, the fraction of all labeled molecules mobile in the time of the experiment. Our spot FPR instrument and software have been described elsewhere (Wolf & Edidin, 1981).

We used a 22× objective producing a e^{-2} spot radius at 1.1 μm for all of the experiments reported here. The measuring laser power density was typically 0.1–1 kW/cm^2; the bleaching power density was 0.2 MW/cm^2. Measurements with a 10X objective affording a spot of 2.4 μm e^{-2} radius yielded D and R values that differed only by 10–20% from those obtained with the smaller spot. This difference is well within the error expected from the accuracy of determining the spot radius. The details of measuring spot radii have been reported earlier (Edidin & Stroynowski, 1991).

In all of the FPR measurements, the signal from liposome samples without fluorescent proteins was 2–4% of the prebleach fluorescence.

Measurement of Rotational Diffusion. Rotational diffusion coefficients were estimated using the technique of time-resolved phosphorescence anisotropy (TPA) (Jovin & Vaz, 1989; Austin et al., 1979; Aroeti et al., 1990; Jovin et al., 1981). Suspensions of erythrosin-HLA-A2-containing liposomes were deoxygenated by purging with a slow flow of nitrogen for approximately 10 min. Deoxygenated sample solutions were resuspended into a nitrogen-saturated 5-mm Suprasil quartz cuvette (Hellma Cells, Inc., Jamaica, NY) mounted in a thermostated holder. The frequency-doubled 532-nm output of a Spectra-Physics DCR-11 Nd–YAG laser provided the excitation pulses for our experiments. The laser was operated at 10 Hz with a vertically polarized TEM 01 output of 1.5 mJ at the sample curvette. Phosphorescence was collected by an f/1.2, 28-mm collector lens system and isolated by a 1 M $Na_2Cr_2O_7$ solution, a KV 550 color filter (Schott Glass Technologies, Inc., Duryea, PA) to block the scattered light from the sample and container, and a 3-mm-thick RG 665 filter to prevent delayed fluorescence from reaching the photomultiplier tube (PMT). Additionally, the system contained a fast gating circuit to turn the PMT off during the periods of intense fluorescence caused by the high-power Nd–VAG pulses. A rotating polarizer was placed in front of the PMT in order to observe the intensity of phosphorescence polarized parallel [$I_\parallel(t)$] or perpendicular [$I_\perp(t)$] to the polarization of the excitation pulse. The phosphorescence signal was collected at 90° to the excitation axis by a thermionically cooled EMI 9816A PMT. The output signal was amplified by a Tektronix 476 oscilloscope whose vertical output was passed through a 35-MHz bandwidth buffer amplifier to a Nicolet 12/70 computer equipped with a 20-MHz analog to digital converter for signal averaging. After data acquisition was complete, the data were downloaded into an Intel 80386-based microcomputer for data analysis and storage. Typically we accumulated three data sets of 1024 cycles for each sample.

Phosphorescence intensities $I_\parallel(t)$ and $I_\perp(t)$ can be analyzed (Jovin & Vaz, 1989; Austin et al., 1979) to yield a phosphorescence intensity function $s(t)$ and phosphorescence anisotropy function $r(t)$. We define s(t) and r(t) as follows:

$$s(t) = I_\parallel(t) + 2gI_\perp(t) \tag{1}$$

$$r(t) = \frac{I_\parallel(t) - gI_\perp(t)}{s(t)} \tag{2}$$

where g is an instrumental constant. In our experiments, $s(t)$ was fitted to a multiexponential decay model to obtain the triplet lifetime(s) of the phosphorescent probe and the amplitude(s) of their decay. Standard errors in these quantities were assigned by the usual methods (Bevington, 1969). Results from the lifetime analysis were used to weight points in a nonlinear least-squares fit of the anisotropy data. Using a weighting factor permitted anisotropy decay data to be properly analyzed according to a single exponential decay model:

$$r(t) = r_\infty + (r_0 - r_\infty)\exp(-t/\phi) \tag{3}$$

Fitting ϕ to eq 3 yielded the initial anisotropy value r_0, the

limiting anisotropy value r_∞, and the rotational correlation time ϕ as well as the statistical uncertainties in these quantities (Bevington, 1969). It should be noted that standard techniques used in nonlinear curve fitting yield uncertainties smaller than the true uncertainties. However, from our available experimental data, true estimates of these uncertainties cannot be assessed.

Flow Cytometric Energy Transfer. An EPICS 752 flow cytometer (Coulter Instruments, Hialeah, FL) was used to measure energy transfer between FITC-labeled (donor) and SR-labeled (acceptor) proteins. R_0 for this pair is 39 Å (J. Hochman and M. Edidin, unpublished).

FCET Measurements on HLA-Containing Liposomes. We excited FITC-labeled proteins with the 488-nm lines of an argon ion laser. This wavelength does not significantly excite the SR-labeled proteins on the surface of small unilamellar liposomes with an average diameter of 0.2–0.4 μm (Mimms et al., 1981). Resonance energy transfer was measured in terms of the quenching of donor fluorescence at 525 nm (Stryer, 1978).

We found, as have others (Childers et al., 1989), that we could reliably detect forward small-angle light-scatter signals from submicrometer-sized liposomes. These signals were used to trigger detection of fluorescence emission at 525 nm from HLA-containing liposomes. Calibration against fluorescent standard beads (Flow Cytometry Standard Corp., Research Triangle Park, NC) and measurement of fluorescence as a function of sample dilution indicated that each fluorescent event detected represented fluorescence from 400 to 1000 HLA-containing liposomes. Despite this, a 10–50-fold dilution of liposome suspension did not significantly alter the measured mean fluorescence. Hence the observed decrease in donor fluorescence measured in liposomes containing donor and acceptor-labeled HLA was not due to dilution errors.

Correction parameters were derived from the data obtained on liposomes containing unlabeled protein or containing only one species (fluorescein-labeled or SR-labeled) of labeled protein. In all cases the lipid/protein ratio was kept constant at 4000:1 for both the MHC molecule and glycophorin. The transfer efficiency, E, is expressed as the ratio of donor fluorescence in liposomes containing both fluorescein- and SR-labeled proteins to donor fluorescence in liposomes containing only fluorescein-labeled proteins (Szollosi et al., 1989).

FCET Measurements of Fab-Labeled JY Cells. Förster-type resonance energy transfer between fluorescein donor and sulforhodamine acceptor Fab fragments of monoclonal antibody BB7.2 (anti-HLA-A2) was studied by flow cytometry (Tron et al., 1984; Matko et al., 1988). Dual laser (488 and 595 nm) excitation was used to determine energy transfer efficiency in labeled cell populations. Before the energy transfer measurements the intensity and specificity of labeling were checked by microscopic observation of membrane (ringlike) staining. The saturability of the staining as well as competition with unlabeled antibody were also tested before Fab conjugates were used for FCET.

Cells were labeled at a high cell density [(2–3) × 10^7/mL] in a small volume with donor + unlabeled Fab, acceptor + unlabeled Fab, or the mixture of donor + acceptor Fab (1:1 or 1:2 molar ratio). The cells were incubated with the premixed antibodies in ice for 45 min and then washed and resuspended in cold PBS at pH 7.4. Labeled samples were stored in ice during the 30–40 min of a measurement. Four intensity histograms were recorded with each labeled cell sample: the fluorescence emission at 525 and 630 nm excited at 488 nm, emission at 630 nm excited at 595 nm, and forward-angle light scattering. Correction factors were determined from fluorescence intensity histograms obtained with single-labeled cells as described by Tron et al. (1984).

Table I: Lateral Diffusion of HLA-A2 in DMPC and SL Liposomes

temp (°C)	$D_{lat} \times 10^9$ [a] (cm²/s)	recovery (%)
	DMPC Liposomes	
21	3.30 ± 0.17	92 ± 1.4
26	5.10 ± 0.48	97 ± 1.3
37	8.20 ± 0.50	98 ± 1.2
	SL Liposomes	
21	5.20 ± 0.26	64 ± 2.2
26	6.40 ± 0.40	70 ± 3.2
37	8.90 ± 0.76	72 ± 2.8

[a] Mean diffusion coefficient ± standard error for at least 20–40 determinations.

RESULTS

Affinity-purified HLA molecules conjugated with fluorescein isothiocyanate (FITC), Fl-HLA, were incorporated into dimyristolphosphatidylcholine, DMPC, or soybean lecithin, SL, liposomes. More than 90% of the protein mixed with DMPC in detergent solution was incorporated in the small liposomes that formed after dialysis. Incorporation of the protein did not affect the pretransition or main transition temperatures of the DMPC liposomes, measured calorimetrically (data not shown).

A substantial fraction of the incorporated HLA molecules was in the same orientation as in the cell membrane, since HLA-containing liposomes reacted with Fab fragments of the HLA-specific mAb BB7.2 but not with Fab of another mAb, 28-14-8, against mouse MHC molecules. The surface density of these molecules, estimated from the fluorescence intensity of bound Fl-Fab fragments, was ~112 molecules/μm², assuming the diameter of liposomes to be 20 μm. This compares with an estimate of 125 molecules of Fl-HLA/μm² made from the fluorescence of vesicles containing Fl-HLA measured in our FPR microscope. The good agreement between the two estimates suggests that the fluorescence measured in FPR comes mainly from labeled HLA molecules in the outer bilayer of multilamellar vesicles.

At 37 °C, lateral diffusion coefficients (D) of Fl-HLA molecules in DMPC liposomes ranged from ~5 × 10^{-9} to 1 × 10^{-8} cm² s^{-1} in different experiments. Mobile fractions (R) were >90%. D and R of the fluorescent lipid analog, NBD-6-PC, were 1 × 10^{-8} cm² s^{-1} and 96%, respectively. D of Fl-HLA in SL liposomes was in the same range but the percent recovery was somewhat lower. D of Fl-HLA increased with increasing temperature in either DMPC or SL liposomes (Table I).

As noted, DMPC liposomes containing unconjugated HLA molecules could be labeled with Fl-Fab. For these Fab-labeled molecules the lateral diffusion coefficient was 4.7 × 10^{-9} cm² s^{-1}; 90% of the label was mobile. These values are almost identical to those for Fl-HLA measured during the same week.

At 37 °C, D and R of FL-HLA were independent of the molar ratio of lipid/protein in a range of 8000:1–2000:1. However, at 21 °C both D and R showed a small, but definite dependence on lipid/protein ratio (Table II). We were unable to form large proteoliposomes at L/P < 2000:1.

D and R were also reduced when the DMPC/HLA mixture was dialyzed for 24 h at 4 °C rather than for 4–5 h. The lateral diffusion coefficient of Fl-HLA in SL liposomes at 37°C was reduced by 40% when the SL/HLA mixture was dialyzed for 24 h. However, in contrast to the results on DMPC liposomes, this decrease in D was reversible. D increased from

Table II: Effect of Density (L/P) on the D_{lat} of HLA-A2

L/P	temp (°C)	$D_{lat} \times 10^9$ (cm^2/s)	recovery (%)
8000	21	1.70 ± 0.08	90 ± 1.0
	37	4.60 ± 0.25	89 ± 2.1
4000	21	1.50 ± 0.12	86 ± 2.6
	37	4.70 ± 0.27	90 ± 1.2
2000	21	0.97 ± 0.05	72 ± 2.7
	37	4.60 ± 0.15	89 ± 2.4

Table III: Effect of Storage of Reconstituted DMPC and SL Liposomes at 4 °C on D_{lat} and Recovery

days	temp (°C)	$D_{lat} \times 10^9$ (cm^2/s)	recovery (%)
	DMPC, L/P Ratio of 4000:1		
1	21	4.20 ± 0.30	94 ± 1.0
	37	13.00 ± 0.82	97 ± 0.7
5	21	2.20 ± 0.22	80 ± 4.6
	37	11.00 ± 1.10	96 ± 0.7
12	21	2.00 ± 0.12	59 ± 3.1
	37	8.90 ± 0.67	89 ± 1.5
	Soybean Lecithin, L/P Ratio of 4000:1		
1	21	5.10 ± 0.20	75 ± 2.4
	37	8.60 ± 0.52	80 ● 1.1
7	21	5.00 ± 0.27	73 ± 2.6
	37	8.40 ± 0.72	80 ± 1.5

(5.2 ± 0.49) × 10^{-9} to (6.9 ± 0.53) × 10^{-9} cm^2 s^{-1} when SL/HLA liposomes were kept at 37 °C for 2 h.

Longer storage at 4 °C further reduced D of Fl-HLA in DMPC liposomes (Table III) but not D of HLA in SL liposomes.

None of the effects of prolonged storage, protein concentration, or temperature were found for Fl-glycophorin. D in DMPC liposomes was 2.5 × 10^{-9} cm^2 s^{-1} at 21 °C and 6.5 × 10^{-9} cm^2 s^{-1} at 37 °C.

The conditions under which D was reduced, prolonged dialysis or storage at 4 °C, favor the dissociation of b2m from HLA heavy chains. In order to see if the decrease in D reflected loss of b2m, we prepared Fl-HLA-containing DMPC liposomes, measured D at 19 °C after 1 day, and then stored aliquots of the remaining liposome suspension in the presence or absence of 12 μM b2m at 4 °C. After 8 days we measured D in each sample at 19 °C. At 1 day D = (7.0 ± 0.50) × 10^{-10} cm^2 s^{-1} measured at 19 °C. After storage of liposomes for 8 days in the absence of b2m, D = (4.5 ± 0.40) × 10^{-10} cm^2 s^{-1}. For liposomes stored in the presence of b2m, D = (6.7 ± 0.60) × 10^{-10} cm^2 s^{-1}. Mobile fractions of all samples were ~90%.

The effects of lipid/protein molar ratio, temperature, and time of storage on the lateral diffusion of Fl-HLA strongly suggested that the population of HLA molecules in the bilayer is a mixture of monomers and oligomers. The proteoliposomes produced by detergent dialysis are large enough (ca. 0.2–0.4 μm average diameter; Mimms et al., 1981) to be detected in a flow cytometer (Childers et al., 1989). Fluorescence resonance energy transfer measurements were performed on such liposomes containing equimolar mixtures of Fl-HLA and sulforhodamine-HLA for a final L/P of 4000:1. At this ratio of donor and acceptor, clustering of HLA molecules in oligomers was most efficiently measured in terms of the quenching of donor (Fl) fluorescence rather than as sensitized acceptor (SR) fluorescence. Figure 2 (top) compares the distribution of donor fluorescence intensities in DMPC vesicles at 37 °C in the absence and presence of acceptor. In the presence of acceptor there is a clear shift in the distribution to lower intensities. In contrast, the intensity distribution of Fl- glycophorin is not changed in the presence of acceptor (Figure 2, bottom). At 37 °C Fl-HLA fluorescence

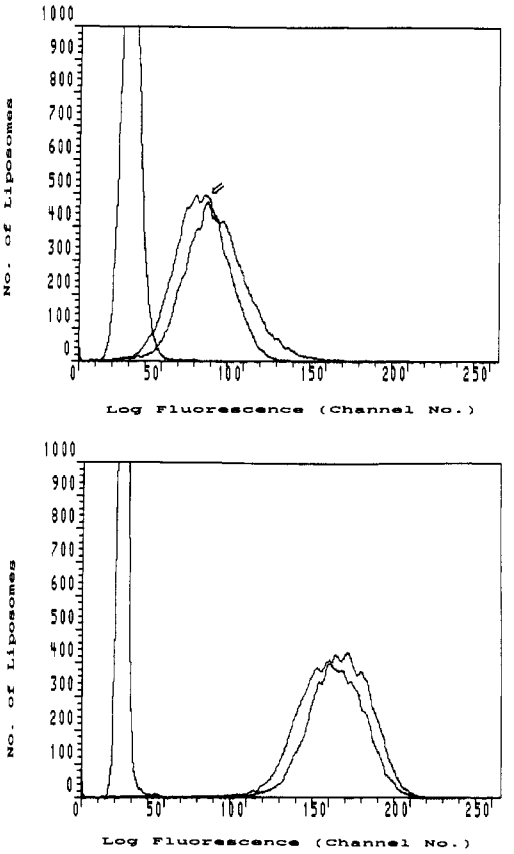

FIGURE 2: (Top) Distribution of green fluorescence in populations of small DMPC liposomes containing either a mixture of Fl-HLA and unlabeled HLA or a mixture of FL-HLA and SR-HLA. The fluorescence of the double-labeled population (arrowed curve) is clearly shifted to lower values. The peak to the far left is autofluorescence from liposomes that have unlabeled HLA. (Bottom) Distribution of green fluorescence in populations of small DMPC liposomes containing either a mixture of Fl-glycophorin and unlabeled glycophorin or a mixture of Fl-glycophorin and SR-glycophorin. There is a small shift in the modal intensity of the double-labeled liposomes, but the distribution pattern is not displaced.

is quenched by SR-HLA an average of 17.0% ± 2.5%. Quenching of glycophorin fluorescence under the same conditions is 4.0% ± 0.40%. Energy transfer between HLA molecules resulted in 26.0% ± 3.0% quenching in SL vesicles at 37 °C (data not shown).

Incubation of liposomes with 2 μM b2m for 2–4 h at 37 °C reduced energy transfer between Fl-HLA and SR-HLA. An example of one experiment is shown in Figure 3. In four separate experiments reductions ranged from 30% to 60% of control. Incubation with micromolar concentrations of two other proteins, cytochrome c and bovine serum albumin, did not reduce energy transfer.

The results from FPR and RET are consistent with the clustering of HLA molecules even in fluid-phase proteoliposomes. We further evaluated the size of the clusters by measuring the rotational diffusion of erythrosin-conjugated HLA molecules using the technique of time-resolved phosphorescence anisotropy. Figure 4 shows the anisotropy decay function for HLA in DMPC liposomes at 30 °C. The rising anisotropy could indicate site-specific labeling of HLA molecules. Rotational correlation times for HLA molecules in DMPC are 239 ± 54 μs at 4 °C, 96 ± 4 μs at 15 °C, and 87 ± 0.8 μs at 30 °C. All these values are much larger than the rotational correlation time expected for a single molecule of HLA in a fluid lipid, namely, ~6 μs.

Rotational correlation times for Er-HLA in liposomes prepared in the presence of 2 μM b2m were about half the

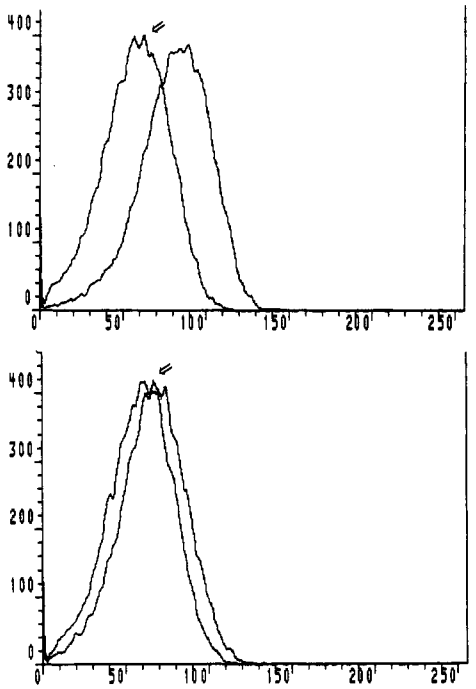

FIGURE 3: Effect of β2-microglobulin, b2m, on energy transfer between Fl-HLA and SR-HLA. The histograms show the distribution of green fluorescence in populations of small DMPC liposomes containing either a mixture of Fl-HLA and unlabeled HLA or a mixture of Fl-HLA and SR-HLA. The fluorescence of the double-labeled population (arrowed curves) is clearly shifted to lower values. The shift is not as great in the presence of b2m (bottom panel) as in its absence (top panel).

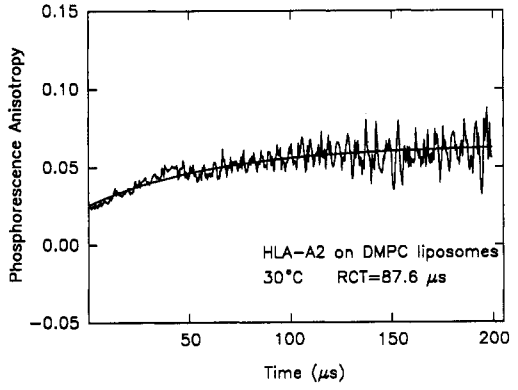

FIGURE 4: Time dependence of anisotropy decay at 30 °C for ErITC-HLA incorporated in DMPC liposomes. The rotational correlation time observed was 87 ± 0.8 μs. The smooth curve is the anisotropy data fitted to a weighted nonlinear least-squares routine.

times measured for Er-HLA in the absence of b2m; 48 ± 2 μs at 25 °C compared to 87 ± 3 μs in the controls (mean ± SEM of six measurements made on two different days).

To see if HLA oligomers are present on cell surfaces we labeled JY cells, human lymphoblasts, with mixtures of Fl- and SR-Fab anti-HLA-A2. There were significant amounts of b2m-free HLA heavy chains, detectable with mAb HC10, on the surfaces of JY cells. Both quenching of donor, Fl-labeled Fab, and sensitized emission from accepor, SR-labeled Fab, were detected by FCET (Figure 5). Neither quenching nor sensitized emission was detected when the surface immunoglobulins (Ig) of JY cells were labeled with Fab of a mouse anti-human Ig (data not shown).

DISCUSSION

HLA-A2 molecules were efficiently reconstituted into proteoliposomes. Approximately 90% of the protein added to DMPC in octyl glucoside solution pelleted with the liposomes

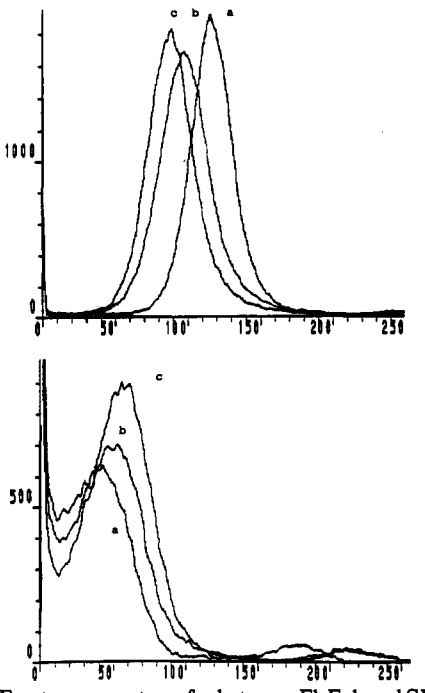

FIGURE 5: Forster energy transfer between Fl-Fab and SR-Fab anti-HLA-A2 bound to JY cells. The flow cytograms display fluorescence intensity—on a 256-channel log scale—on the abscissa and number of cells on the ordinate. The top panel shows donor fluorescence excited at 488 nm and measured at 510–540 nm in the presence of (a) Fl-Fab + unlabeled Fab, (b) 1:1 Fl-Fab/Sr-Fab, and (c) 1:2 Fl-Fab/SR-Fab. Notice that the distribution of fluorescence intensities shifts to lower values as the concentration of acceptor increases. The bottom panel shows acceptor fluorescence excited at 488 nm and measured at 620–650 nm in the presence of (a) SR-Fab + unlabeled Fab, (b) 1:1 SR-Fab/Fl-Fab, and (c) 2:1 SR-Fab/Fl-Fab. Notice that the distribution of fluorescence intensities shifts to higher values as the concentration of acceptor increases. The number of cells in the peak also increases as their fluorescence exceeds that of background (<channel 10). The small peaks to the right are caused by fluorescent debris and cell aggregates.

formed after dialysis. The HLA molecules were largely incorporated with their large extracellular domains facing the solution. The number of Fl-Fab bound per unit area of proteoliposome corresponded to the number of HLA per unit area calculated from the input lipid/protein molar ratio.

Though D varied from batch to batch of liposomes, in a given experiment HLA molecules in DMPC liposomes diffused to nearly the same extent at 19–21 °C, below the phase transition, as they did at 37 °C. However, the presence of the protein did not alter the phase transition temperature of the liposomes (23–24 °C). This effect has also been reported for glycophorin incorporated into DMPC (Vaz et al., 1981). It has been ascribed to an "icebreaker" effect of the transmembrane portion of the glycoprotein, locally perturbing the surrounding gel phase. This effect is also consistent with our observation that the rotational diffusion of HLA was hardly changed when the temperature was lowered from 30 to 15 °C.

At high L/P, ~95% of the labeled HLA molecules were mobile in the plane of DMPC liposome membranes over the temperature range 21–37 °C. Surprisingly, a significantly smaller fraction, ~75%, of HLA was mobile in SL liposomes, possibly because these liposomes were smaller than DMPC liposomes. The smaller the liposome, the greater fraction of the total fluorescence is bleached and the lower the possible recovery.

Published work on diffusion of bacteriorhodopsin in liposomes suggested that protein concentration has little effect on D_{lat} and R when the protein concentration is <1 mol % with respect to the bulk phospholipid (Peters & Cherry, 1982).

However, we found that D and R of HLA were reduced when the concentration of HLA molecules was as small as 0.05% (L/P 2000:1). D and R were reduced both at 21 and 37 °C when DMPC proteoliposomes were stored below their phase transition, at 4 °C for 1–12 days (Table III). Storage of large SL proteoliposomes for 1 day at 4 °C also reduced D of HLA, while prolonged (2 h) incubation of these liposomes at 37 °C significantly increased D. In contrast, there was no change in D or R when glycophorin/DMPC proteoliposomes were stored for up to 20 days (data not shown).

Vaz et al. (1981) and we (Table III) found that D_{lat} for glycophorin in DMPC increased no more than 2-fold when the temperature was increased from 20 to 40 °C. The change in D_{lat} of HLA in SL liposomes was also about 2-fold going from 21 to 37 °C. In contrast, D_{lat} of HLA in DMPC increased 2–5-fold in this range. The largest increases in D_{lat} were found after the proteoliposomes had been stored for 5 or 12 days. These results reinforce a model in which HLA aggregates in gel-phase lipid diffuse less readily than HLA monomers.

If HLA molecules form clusters, then we expect to detect resonance energy transfer between molecules labeled with donor and acceptor fluorophores. Flow cytometry has previously been used to measure energy transfer in populations of membrane vesicles (Gorvel et al., 1984, 1989; Tron et al., 1984; Matko et al., 1988). We found that we could measure fluorescence in the small proteoliposomes formed by detergent dialysis, made of fluorescent HLA-A2 or glycophorin and DMPC or SL. We readily detected energy transfer, measured as significant quenching of donor fluorescence, between Fl- and SR-labeled HLA even at 37 °C. There was only slight quenching of donor fluorescence in glycophorin-containing liposomes. This may reflect some degree of oligomerization of glycophorin (Romans, 1978; Vaz et al., 1981), but it is well within the error of the fluorescence measurements.

Resonance energy transfer is a sensitive qualitative method for detecting molecular aggregation, but it does not define the cluster size. To this end we measured the rotational diffusion of erythrosin-labeled HLA. At 15 and 30 °C the measured rotational correlation times are very much longer than those estimated for glycophorin (van Hoogevest et al., 1985) and they are 20–50-fold greater than those reported for the well-characterized membrane protein bacteriorhodopsin (Cherry & Godfrey, 1981). If we neglect any effects of the exodomains of HLA on its rotation, this implies aggregates with radii ~4–7 times that of a monomer.

Saffman and Delbruck (1975) and Hughes et al. (1982) predicted that while $D_{rot} \propto r^2$, $D_{lat} \propto \ln r$. This seems to be correct for a series of membrane proteins differing about 8-fold in molecular weight or ~2-fold in radius (Vaz et al., 1982). The size of the HLA-A2 aggregates at 37 °C in SL liposomes may be conservatively estimated from the ratio of D_{lat} measured at 37 °C in 24-h-old SL liposomes, $(5.2 \pm 0.26) \times 10^{-9}$ cm^2 s^{-1}, to D_{lat} measured in 5-h-old SL liposomes, $(8.6 \pm 0.52) \times 10^{-9}$ cm^2 s^{-1}. Assuming that the higher value represents diffusion of monomeric HLA, we estimate the aggregate radius as $e^{8/5} r_{monomer}$ or about 5 times $r_{monomer}$. This is in good agreement with the estimate of aggregate size from the rotational diffusion measurements.

Comparing the results on HLA in DMPC liposomes and those on HLA in SL liposomes shows that the accumulation of HLA aggregates over time depends on the composition, and presumably the phase state, of the liposome bilayer. However, the aggregates are not artifacts of reconstitution of purified HLA into liposomes. HLA aggregates were also detected by resonance energy transfer on the surface of human B-lymphoblastoid JY cells. These cells are rich in class I molecules, bearing on the order of 10^6 molecules/cell, but the energy transfer observed is not likely to be due to a high average density of class I molecules. The surface area of a JY cell, approximated as a sphere of 8-μm radius, is ~800 μm^2. This yields an average density of HLA of 1/80 000 Å^2; class I molecules are separated by hundreds of angstroms on the cell surface. Consistent with this calculation, we could not detect energy transfer between Fab-labeled surface Ig on JY. The surface density of these molecules is about the density of HLA.

Our results on JY are consistent with results in HLA-containing liposomes and are concordant with recent reports that dimeric and tetrameric class I molecules can be isolated from a number of mouse cell lines (Krishna et al., 1992; Capps et al., 1992). However, they are contradicted by a published report that FCET between class I HLA molecules could not be detected on JY cells (Szollosi et al., 1989). One of these authors has since been able to measure FCET between class HLA molecules (J. Szollosi, personal communication). It may be that culture conditions affect the fraction of clustered HLA molecules on a given cell type.

A native class I MHC molecule competent to present antigen consists of a trimolecular complex of the 45-kDa heavy chain, the 11-kDa light chain, β2-microglobulin, and a peptide (Bjorkman et al., 1987; Saper et al., 1991). The conditions that we found to enhance aggregation of HLA-A2 molecules in liposomes are also conditions under which the light chain, b2m, can dissociate.

Addition of b2m to the liposomes inhibits HLA aggregate formation measured by all three of the physical methods used by us, FPR, FCET, and TPA. b2m-associated inhibition of aggregate formation appears to be total when measured by our least sensitive method, FPR, and partial when measured by the other two methods. The persistence of HLA aggregates in the presence of excess b2m may reflect the extent of accessibility of free HLA heavy chains to b2m or may be due to persistence of aggregates even after all of their component HLA molecules are associated with b2m.

The b2m is important in maintaining the conformation and the function of class I molecules. The complex of peptide with heavy chain appears to be stabilized by b2m (Tsomides et al., 1991) and may disassemble if b2m dissociates (Rock et al., 1991; Boyd et al., 1992). The population of free heavy chains formed in this way is unable to function in antigen presentation (Rock et al., 1991).

The dissociation of b2m the HLA heavy chain is common. Substantial amounts of free heavy chain are detectable at the surface of human and mouse cells, including activated normal T-cells (Schnabl et al., 1990; Madrigal et al., 1991; Rock et al., 1991; Lie et al., 1991). There is considerable debate on the function of these free heavy chains. One view has it that they are rapidly degraded and remove class I molecules from the presenting population (Rock et al., 1991). Another view is that free class I heavy chains may bind exogenous peptide from solution and reassemble together with exogenous (plasma) b2m (Madrigal et al., 1991; Benjamin et al., 1991; Luescher et al., 1991). A recent report suggests that b2m-free MHC molecules function in T-cell regulation (Demaria et al., 1992); they cap more quickly and efficiently than do b2m-associated MHC molecules.

The possibility has also been raised that b2m-free HLA heavy chains can associate with other proteins (Bushkin et al., 1988; Schnabl et al., 1990). This suggestion is consistent with our observations of HLA-A2 aggregates on the surfaces of intact human JY lymphoblasts. JY cells express a substantial fraction of HLA lacking b2m, yet our measurements were made with Fab fragments of an mAb against

native, b2m-containing HLA-A2. We have also been able to detect clustering using Fab of another mAb of this type, W6/32 (J. Matko and M. Edidin, unpublished). These observations imply that clusters of HLA-A2 contain both b2m-free and b2m-containing HLA molecules. Complexes of this sort, aggregates of antigen-presenting HLA trimers, could provide a more effective stimulus to T-cells than HLA monomers since in virus-infected cells they are liable to include multiple copies of the same antigenic peptide. Cells would be protected from efficient sensitization by exogenous peptides if the aggregates dispersed when b2m-free molecules took up the peptides. These speculations can be tested by examining the effect of added b2m on HLA aggregation and sensitivity of cells to T-cell effectors.

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