Lateral Mobility of Class I Histocompatibility Antigens in B Lymphoblastoid Cell Membranes: Modulation by Cross-linking and Effect of Cell Density

Barbara E. Bierer,*‡ Steven H. Herrmann,‡‖ Carl S. Brown,*§ Steven J. Burakoff,‡¶ and David E. Golan*§

*Division of Hematology, Department of Medicine, Brigham and Women's Hospital;
‡Division of Pediatric Oncology, Dana-Farber Cancer Center; and the Departments of *Medicine, ‖Pathology, ¶Pediatrics, and §Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Abstract. We have studied the lateral mobility of class I major histocompatibility complex (MHC) proteins in the membranes of human Epstein-Barr virus–transformed B cells using fluorescence photobleaching recovery. Class I MHC antigens were labeled with either W6/32 monoclonal antibody or its Fab fragment directly conjugated to fluorescein isothiocyanate. The diffusion coefficient of class I antigens labeled with Fab fragments of W6/32 was identical to that of a lipid analogue, fluorescein phosphatidylethanolamine, and was 10-fold greater than that of antigens labeled with intact W6/32. Furthermore, antigens labeled with Fab fragments but not with intact W6/32 had fractional mobilities identical to that of the lipid probe. The lateral mobility of class I antigens was dependent on the time of incubation with fluorescent antibody and on the presence of antibody microaggregates. Finally, class I MHC proteins labeled with intact W6/32 but not with Fab fragments were immobilized in the membranes of most cells grown in suspension at high cell density. These results suggest that, in the unperturbed state, class I MHC antigens diffuse as rapidly as membrane lipid, i.e., without cytoskeletal constraint. Cross-linking with bivalent ligand and growth to high cell density may trigger membrane events leading to slowing and immobilization of these proteins.

HUMAN class I major histocompatibility complex (MHC)[1] antigens comprise a family of polymorphic transmembrane cell surface glycoproteins present on all nucleated cells. The prototype molecule consists of a polymorphic 44-kD heavy chain that is noncovalently associated with an invariant 12-kD light chain, β_2 microglobulin. The heavy chain is comprised of a glycosylated extracellular domain (~270 amino acid residues), a hydrophobic transmembrane segment (~25 residues), and a hydrophilic intracytoplasmic domain (~30 residues) (16). Proteins encoded by the MHC are important recognition structures that control the cellular immune response. T cell specific receptors recognize MHC alloantigens or syngeneic MHC proteins in association with foreign antigens (30). The lateral mobility of MHC proteins in target cell membranes may affect recognition of these cells by T cells. In addition, the mobility of these proteins may reflect interactions with cell surface receptors (10, 26, 29) and/or cytoskeletal proteins (13, 39). Such interactions may be important in signal transduction across cell membranes (1).

Literature values for the lateral mobility of class I MHC proteins in biological membranes are variable. Human and mouse class I MHC antigens in lymphocyte and fibroblast cell membranes are reported to have fractional mobilities (f) of 0–80% and diffusion coefficients that range from 2×10^{-10} to 2×10^{-9} cm^2 s^{-1} (6, 8, 9, 13, 25, 31, 38, 39). In general, these diffusion coefficients are one to two orders of magnitude less than those of lipid probes in the same cell membranes (7, 13, 25). The variation in lateral mobility has not been fully explained, although cytoskeletal interactions have been invoked (13, 39).

We have examined the effects of mAb valency and of cell density on the lateral mobility of class I MHC antigens in plasma membranes of Epstein-Barr virus (EBV)–transformed human B cells. Class I human leukocyte antigens (HLA) were labeled with fluorescein conjugates of either W6/32, a mAb that binds to a monomorphic determinant on all human class I antigens (24), or Fab fragments of W6/32. The lateral mobility of labeled molecules was measured by fluorescence photobleaching recovery (FPR). We observed that the valency of the mAb used for labeling influenced class I MHC antigen mobility. MHC antigens labeled with Fab fragments of W6/32 diffused as fast as a phospholipid analogue in the same cell membrane, and ten times faster than MHC antigens labeled

1. *Abbreviations used in this paper*: EBV, Epstein-Barr virus; f, fractional mobility; Fl-PE, fluorescein phosphatidylethanolamine; FPR, fluorescence photobleaching recovery; HLA, human leukocyte antigens; MHC, major histocompatibility complex.

with intact W6/32. Furthermore, MHC antigens labeled with Fab fragments but not with intact W6/32 had fractional mobilities identical to lipid. Microaggregates of intact IgG or Fab fragments, as well as increasing time of incubation with antibody, each caused the lateral mobility of class I MHC molecules to decrease. Finally, class I MHC antigens labeled with intact W6/32 but not with Fab fragments were immobilized in the membranes of most cells grown at high cell density.

Materials and Methods

Cell Lines and Culture

The EBV-transformed B cell line JY (HLA A2,2; B7,7; DRw4,w6; DQ1,3; DP2,4) was maintained in RPMI-1640 medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 10 mM Hepes (M. A. Bioproducts), 2 mM glutamine (Gibco), and 25 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). Unless otherwise stated, cells were split 1:10 every 3 d to maintain log phase of growth.

Purification and Digestion of Monoclonal Antibodies

The mAb W6/32 (the kind gift of Dr. Peter Parham [Stanford University, Stanford, CA], and American Type Culture Collection, Rockville, MD) recognizes a monomorphic determinant on HLA-A,B,C molecules (24). The antibody was purified from ascitic fluid of pristane-primed BALB/c mice by passage over a protein A-Sepharose 4B (Pharmacia Fine Chemicals, Div. Pharmacia, Inc., Piscataway, NJ) column at pH 9.0 and eluting from the washed column at pH 4.0, as described (22). Monovalent Fab fragments were prepared from purified W6/32 by papain digestion (22). Papain (Sigma Chemical Co.), 1 mg/ml, was activated by incubation in 100 mM sodium acetate, 100 mM dithiothreitol, 100 mM EDTA, pH 5.5, for 10 min. W6/32, 1 mg/ml, was added and the mixture incubated at 37°C for 1 h. The reaction was stopped by addition of 25 mM iodoacetic acid. Alternatively, papain, 5 mg/ml, was coupled to Sepharose 4B. After activation with dithiothreitol, the papain-coupled beads were washed three times in 100 mM sodium acetate buffer and then added to purified antibody, 5 mg mAb per ml of papain beads. The mixture was incubated at 37°C for 6 h. Protease-modified antibody was passed over a protein A-Sepharose 4B affinity column which bound the Fc fragment and any remaining intact antibody. Fractions containing the nonbinding Fab fragment were assessed by Coomassie Blue and silver staining (Bio-Rad Chemical Division, Bio-Rad Laboratories, Richmond, CA) (21) after electrophoresis on a 5–15% linear gradient SDS polyacrylamide gel (17). Under nonreducing conditions, a single protein band at M_r 50,000 was seen; under reducing conditions, two protein bands with M_r ~25,000 were observed. Protein concentrations were determined by the method of Lowry (18).

Conjugation of Fluorescein to Monoclonal Antibodies

Intact mAb and Fab fragments were conjugated to FITC (Sigma Chemical Co.) as described (22). Fluoresceinated antibodies were dialyzed against PBS, 10 mM glycine, pH 7.2, and exhaustively against PBS. Alternatively, FITC was dissolved in dimethyl sulfoxide (Fisher Scientific Co., Fair Lawn, NJ) at 3 mg/ml. Four 10-μl aliquots of this mixture were added at 5-min intervals at 4°C to a 0.67 mg/ml solution of W6/32-Fab in 100 mM carbonate buffer, pH 9.2. After a 2-h incubation at 23°C, the mixture was applied to a Bio-Gel P-100 (Bio-Rad Laboratories) column eluted with PBS, pH 7.2. To remove antibody aggregates, labeled antibodies were centrifuged at 100,000 *g* (Airfuge; Beckman Instruments, Inc., Palo Alto, CA) for 20 min, and the antibody remaining in the supernatant was stored at −20°C. mAb was again centrifuged at 100,000 *g* for 20 min within 6 h of use in FPR experiments, unless otherwise stated.

Labeling of Cells with Fluorescent Antibodies and a Lipid Analogue

The incorporation of the phospholipid analogue fluorescein phosphatidylethanolamine (Fl-PE) into cell membranes has been described (12). Briefly, 10–40 μl of Fl-PE, 200 μg/ml in PBS, was added to 400 μl of JY cells, 1–5 × 10^5 cells/ml. The mixture was incubated in the dark at room temperature for 30 min, washed twice in PBS containing 1% BSA, and once in Dulbecco's PBS supplemented with 2% BSA, 1% essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10 mM glucose (D-PBS/BSA). The lateral diffusion rate of Fl-PE in JY cell membranes was constant for labeling concentrations of 5–20 μg/ml. In fluorescent antibody experiments, 5 μl of antibody, 0.5 mg/ml, was incubated with 150 μl of cells, 5 × 10^5 cells/ml, in the dark on ice for 20 min, and the cells were washed twice at 4°C. Washed, labeled cells were resuspended in 100 μl D-PBS/BSA, and 5 μl of suspension was placed on a PBS/BSA-coated microscope slide, covered with a PBS/BSA-coated coverslip, and sealed with vacuum grease (12). Unless otherwise stated, cells were used in FPR experiments within 30 min of labeling, and measurements were completed within 60 min of labeling.

Fluorescence Photobleaching Recovery

Our FPR apparatus and analytical methods have been described in detail (12). FPR is a technique used to measure the lateral mobility of fluorescently labeled proteins and lipids in membranes (2). Briefly, a Gaussian laser beam is focused to a waist at the sample plane of a fluorescence microscope. After a brief, intense photobleaching pulse, the recovery of fluorescence is measured. Recovery results from the lateral diffusion of unbleached molecules into the bleached area. Nonlinear least squares analysis of fluorescence recovery curves yields both the fractional recovery (f), which represents that fraction of labeled molecules free to diffuse laterally on the time scale of the experiment, and the diffusion coefficient (D) of the mobile fraction (12). The Gaussian beam radius at the sample plane was determined daily as described (12), and its mean value was between 0.8 and 1.2 μm with SD < 5%. The photobleaching power at the sample was ~100 μW, and the measuring beam intensity was ~0.2 μW. The bleaching time was typically 25 ms for diffusion measurements on cells labeled with intact W6/32, and 5 ms for experiments on cells labeled with Fab fragments or Fl-PE. Fluorescence in the medium was <2% of cell-associated fluorescence for cells labeled with intact mAb or Fl-PE, and 2–10% of membrane fluorescence for Fab fragment–labeled cells, and this background was subtracted from the measured fluorescence. Autofluorescence, determined with unlabeled cells, was <1% of the fluorescence of all labeled cells. Temperature was controlled to 23 ± 0.1°C by a thermal microscope stage.

Unless otherwise stated, experiments were repeated at least three times each on different days. Results of experiments performed under identical conditions were pooled unless significant differences between the results on different days were observed.

Results

Fl-PE Distribution and Lateral Mobility

By fluorescence microscopy, Fl-PE was uniformly distributed on the surface of JY cells, an EBV-transformed B lymphocyte cell line. The diffusion coefficient at room temperature was 2.3 ± 0.2 × 10^{-9} cm^2 s^{-1} (mean ± SEM), and the fractional mobility was 89 ± 3% (n = 27) (Table I). The diffusion coefficient at 37°C was 4.9 ± 0.8 × 10^{-9} cm^2 s^{-1}, and the fractional mobility was 93 ± 2% (n = 8).

Intact IgG Binding Restricts the Lateral Mobility of Class I MHC Antigens

JY cells were labeled with fluorescein-conjugated W6/32 mAb. As shown in Table I, the diffusion coefficient of W6/32-FITC–labeled proteins was 3.2 ± 0.5 × 10^{-10} cm^2 s^{-1}, and the fractional mobility was 54 ± 3% (n = 128). Class I HLA antigens were also labeled with Fab fragments prepared from W6/32. The diffusion coefficient of FITC-W6/32-Fab–labeled antigens was 2.1 ± 0.2 × 10^{-9} cm^2 s^{-1}, and the fractional mobility was 90 ± 2% (n = 63). The binding of each probe was blocked by excess unlabeled W6/32. Both W6/32 and its fluoresceinated Fab fragment immunoprecipi-

Table I. Lateral Mobility of Class I MHC Antigens Labeled with Intact W6/32, W6/32 Fab Fragments, and a Phospholipid Analogue in JY Cells

Fluorophore*	D	f	N
	($\times 10^{10}$ cm^2 s^{-1})	%	
W6/32-FITC	3.2 ± 0.5‡[1]	54 ± 3[2]	128
FITC-W6/32-Fab	21 ± 2[1,3]	90 ± 2[2,4]	63
FITC-W6/32-Fab$^+$ (Fab)'$_2$ GAM	1.3 ± 0.3[3]	59 ± 6[4]	18
F1-PE	23 ± 2	89 ± 3	27

D, diffusion coefficient; f, fractional mobility; N, number of measurements.
* Fluorophore centrifuged at 100,000 g for 20 min within 6 h of use in FPR experiments.
‡ Mean ± SEM.
[1,2,3,4] $P < 0.001$, Student two-tailed t test.

Table II. Effect of mAb and Fab Fragment Centrifugation on the Lateral Mobility of Class I MHC Antigens

Fluorophore	Centrifugation	D	f	N
		($\times 10^{10}$ cm^2 s^{-1})	%	
W6/32-FITC	9,000 g, 20 min	5.2 ± 1.8[1]	51 ± 10[2]	8
	100,000 g, 20 min	9.6 ± 1.8[1]	88 ± 7[2]	5
FITC-W6/32-Fab	9,000 g, 20 min	6.3 ± 1.3[3]	98 ± 2[4]	8
	100,000 g, 20 min	17.7 ± 3.2[3]	79 ± 12[4]	4

D, diffusion coefficient; f, fractional mobility; N, number of measurements.
* Mean ± SEM.
[1,4] $P > 0.05$, Student two-tailed t test.
[2] $P < 0.05$, Student two-tailed t test.
[3] $P < 0.01$, Student two-tailed t test.

tated two polypeptide proteins, of M_r 44,000 and 12,000, from the surface of radioiodinated JY cells. These molecular weights correspond to those of the HLA heavy chain and β_2 microglobulin, respectively. By analysis on a fluorescence activated cell sorter (FACS II; Becton-Dickinson & Co., Mountain View, CA), W6/32-FITC and FITC-W6/32-Fab each labeled JY cells brightly, but failed to label Daudi (HLA A-,B-,C-; DRw6, DQ1, DP2,4) and K562 (HLA A-,B-,C-; DR-,DQ-,DP-), two cell lines that do not express class I HLA molecules. These data suggest that the fluoresceinated Fab fragment of W6/32 binds specifically to HLA-A,B,C and not to a lipid component on the cells. No diminution in the fluorescence intensity of FITC-W6/32-Fab–labeled cells was observed over the 60-min FPR experimentation period, implying the fluoresceinated Fab fragment binding is stable as well as specific.

In an attempt to mimic the effect of bivalent antibody, cells were labeled with FITC-W6/32-Fab and then incubated in the dark for 20 min on ice with a fluoresceinated (Fab)'$_2$ goat anti-mouse antibody fragment. The second antibody fragment was titrated up to a concentration at which the fluorescence of the doubly labeled cells was just increased over that of cells labeled with FITC-W6/32-Fab. Both the diffusion coefficient and fractional mobility of the doubly labeled class I MHC proteins decreased to values similar to those observed on cells labeled with W6/32-FITC (Table I). Taken together, these data suggest that antigen cross-linking by bivalent mAb induces constraints on the mobility of class I MHC antigens.

Lateral Mobility of Class I Antigens Is Restricted by Microaggregates of Intact W6/32 or W6/32 Fab Fragments

mAb was centrifuged for 20 min at either 9,000 g (Microfuge; Eppendorf; Brinkmann Instruments, Inc., Westbury, NY) or 100,000 g (Airfuge; Beckmann Instruments, Inc.) within 6 h of use in FPR experiments. Cells in log phase of growth were labeled with mAb. The fractional mobility of class I MHC antigens labeled with W6/32-FITC was significantly increased by centrifugation of antibody at 100,000 g for 20 min (Table II). In addition, a time dependent decrease in both diffusion coefficient and fractional mobility was observed upon incubation of cells with W6/32-FITC that had been centrifuged at 9,000 g for 20 min (Fig. 1). Antibody centrifuged at 100,000 g for 20 min did not show this time dependence. The diffusion coefficient of proteins labeled with FITC-W6/32-Fab was significantly increased by centrifugation of antibody at 100,000 g for 20 min, and the fractional mobility of these proteins was maximal under both centrifugation conditions (Table II). The cell surface fluorescence distribution was uniform on cells labeled with W6/32-FITC or FITC-W6/32-Fab that had been centrifuged at either 9,000 or 100,000 g and incubated with JY cells on ice for 20 min, and this distribution did not change over the 60-min FPR experimentation period at room temperature.

Lateral Diffusion of Class I Antigens Is Dependent on the Time of Incubation with W6/32 or Its Fab Fragment

The time of incubation with both intact mAb and Fab fragments influenced the lateral mobility of class I MHC antigens. Antibodies centrifuged at 100,000 g were used in this study. 4-h incubation with intact W6/32 resulted in nearly complete immobilization of class I MHC antigens, while 6-h incubation with Fab fragments caused a significant decrease in the fractional mobility but not the diffusion coefficient. Data from representative experiments are shown in Table III. These observations suggest that constraints on protein mobility may be induced by monovalent as well as divalent ligand binding. By fluorescence microscopy, punctate fluorescence was observed after a 4-h incubation with

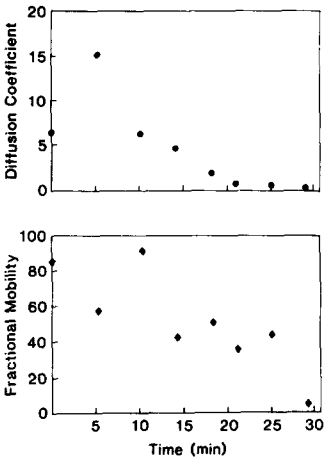

Figure 1. Diffusion coefficient (*top*), ($\times 10^{10}$ cm^2 s^{-1}) and fractional mobility (*bottom*) (%) of class I MHC antigens labeled with W6/32-FITC, as a function of time of incubation. W6/32-FITC was centrifuged at 9,000 g for 20 min immediately before cell labeling. JY cells in log phase of growth were labeled with W6/32-FITC for 20 min at 0°C, washed twice at 4°C, and resuspended at room temperature in D-PBS-BSA (see Materials and Methods). FPR was performed at 23°C. Elapsed time between resuspension of the cells in D-PBS/BSA and the first lateral mobility measurement ($t = 0$) was ~5 min.

Table III. Effect of Time of Incubation with mAb or Fab Fragments on Lateral Mobility of Class I MHC Antigens

Fluorophore*	Incubation time	D	f	N
	h	($\times 10^{10}$ cm^2 s^{-1})	%	
W6/32-FITC	0	10 ± 5‡	77 ± 5[1]	8
	4	–§	18 ± 10[1]	4
FITC-W6/32-Fab	0	23 ± 6[2]	89 ± 4[3]	13
	6	25 ± 8[2]	40 ± 11[3]	7

D, diffusion coefficient; f, fractional mobility; N, number of measurements.
* Fluorophore centrifuged at 100,000 g for 20 min within 6 h of use in FPR experiments.
‡ Mean ± SEM.
§ Diffusion coefficient cannot be measured for $f < 20\%$.
[1,3] $P < 0.001$, Student two-tailed t test.
[2] $P > 0.05$, Student two-tailed t test.

Table IV. Effect of Cell Density on the Lateral Mobility of Class I MHC Antigens

Fluorophore*	Cell growth	D	f	N
		($\times 10^{10}$ cm^2 s^{-1})	%	
W6/32-FITC	Log phase	6.0 ± 2.2	67 ± 7[1]	19
	High density	–	12 ± 3[1]	20
FITC-W6/32-Fab	Log phase‖	17 ± 2	89 ± 3	11
	High density‖	18 ± 5	101 ± 5	8
	Log phase¶	19 ± 3	95 ± 4	8
	High density¶	16 ± 2	89 ± 6	8

D, diffusion coefficient; f, fractional mobility; N, number of measurements.
* Fluorophore centrifuged at 100,000 g for 20 min within 6 h of use in FPR experiments.
‡ Mean ± SEM.
§ Diffusion coefficient cannot be measured for $f < 20\%$.
‖ Cells labeled with 20 μl FITC-W6/32-Fab.
¶ Cells labeled with 10 μl FITC-W6/32-Fab.
[1] $P < 0.001$, Student two-tailed t test.

intact mAb. The fluorescence pattern on Fab-labeled cells, in contrast, remained uniform at 6 h.

Fractional Mobility of Class I Antigens Labeled with Intact W6/32 Is Dependent on Cell Density

Cell density profoundly influenced the lateral diffusion of class I HLA antigens labeled with bivalent mAb. Cells were maintained in log phase of growth, 1–5 × 10^5 cells/ml, by serially splitting the culture 1:10 every 2–3 d, and 1 d before FPR measurements. Alternatively, cells were grown at high cell density, 2–3 × 10^6 cells/ml, by exchanging the growth medium every day for 4 d before FPR measurements. Both log phase and high density cell preparations were 100% viable by trypan blue exclusion. Cells were labeled with identical preparations of W6/32-FITC or FITC-W6/32-Fab. The fractional mobility of class I HLA antigens labeled with bivalent antibody in cells maintained in log phase of growth was 67 ± 7% (n = 19), whereas the fractional mobility of similarly labeled antigens in cells grown at high cell density was 12 ± 3% (n = 20) (Table IV). The distribution of the fractional mobility data is shown in Fig. 2. There was no significant difference in the fluorescence intensity of cells in the two populations, and it is therefore unlikely that the difference in fractional mobility can be explained by differential expression of class I HLA antigens. Cell density did not affect the diffusion coefficient and fractional mobility of class I HLA proteins labeled with Fab fragments of W6/32 (Table IV).

Discussion

In this report, we have shown that Fab fragment-labeled class I HLA molecules diffuse as rapidly and as completely as a lipid analogue, Fl-PE, in plasma membranes of human EBV-transformed B cells. The diffusion coefficient of class I MHC antigens is reduced by an order of magnitude when the molecules are cross-linked either by intact mAb or by Fab fragments and second antibody. Studies using competition with unlabeled antibody, immunoprecipitation, fluorescence-activated cell sorter analysis, and fluorescence microscopy suggest that Fab fragment binding is both specific and stable. The measured diffusion coefficient of Fab fragment-labeled proteins, 2.1 × 10^{-9} cm^2 s^{-1}, and fractional mobility, 90%, therefore reflect the mobility of the class I MHC molecules and not that of fluorophore bound nonspecifically to the membrane. In addition, the concordance between the lateral mobility of Fab fragment-labeled molecules and Fl-PE suggests that class I MHC molecules, under these conditions, are constrained in their mobility only by forces intrinsic to the lipid bilayer itself. Cross-linking of class I molecules may induce a change in cytoskeletal or membrane skeletal organization which results in significant slowing and immobilization of these transmembrane proteins.

Our results are similar to measurements of histocompatibility antigen mobility in membranes of rat spleen cells in suspension labeled either with Fab fragments alone or with Fab fragments and second F(ab)'$_2$ antibody fragments (39). Other studies have reported no apparent dependence of histocompatibility antigen mobility on antibody valence, although the purity and aggregation state of Fab fragments and the cell density used in these studies were not discussed (6, 9, 31). Smith et al. found that the diffusion coefficient of class I HLA antigens on the surface of JY cells ranged from 3.5 to 10.9 × 10^{-10} cm^2 s^{-1}, depending on the particular mAb used to label HLA molecules (31). This threefold variation

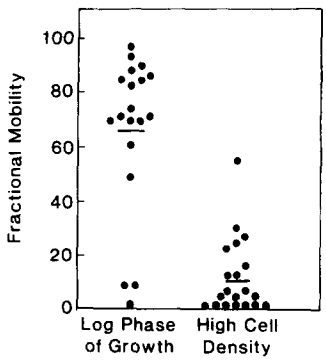

Figure 2. Fractional mobility (%) of class I MHC antigens labeled with W6/32-FITC, as a function of cell density. JY cells were maintained in log phase of growth, 1–5 × 10^5 cells/ml, by serially splitting the culture 1:10 every 2–3 d, or maintained at high cell density, 2–3 × 10^6 cells/ml, by exchanging the growth medium every day for 4 d, before FPR measurements. Cells were washed, resuspended at 5 × 10^5 cells/ml, and labeled with W6/32-FITC for 20 min. W6/32-FITC was centrifuged at 100,000 g for 20 min immediately before use. Cells were again washed and resuspended, and FPR was performed at 23°C. The fractional mobility of class I MHC antigens in cells in log phase of growth, 67 ± 7% (mean ± SEM), was significantly different from that in cells grown at high cell density, 12 ± 3% ($P < 0.001$, Student two-tailed t test).

in diffusion coefficient is consistent with our observation that bivalent mAb labeling may itself perturb HLA mobility.

The diffusion coefficient of Fl-PE in the present study is lower than that observed in studies on JY cells using the lipid probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (25). This difference may reflect real differences in the lipid environment sampled by the probes. Alternatively, it may be due to disruption of membrane architecture by the carbocyanine probe itself (3, 12, 14).

Theoretical and experimental considerations support the conclusion that Fab fragment–labeled class I MHC molecules are free from protein–protein interactions that could retard lateral diffusion. The limiting diffusion rate of a transmembrane protein is thought to be determined by interactions between the membrane-spanning region of the protein and membrane bilayer lipid. Such interactions depend on the size of the transmembrane segment (28, 36, 37). Assuming that the membrane-spanning portion of a class I HLA molecule consists of a single α-helical segment, the diffusion rate of this molecule, in the absence of extrabilayer interactions, should be approximately equal to that of a phospholipid molecule (11, 28). The M13 phage coat protein has a single membrane-spanning domain consisting of ~20 amino acid residues, similar to class I HLA molecules. In fluid-phase model membranes containing only phospholipid, cholesterol, and reconstituted M-13 coat protein, the diffusion of this protein is as rapid as that of a phospholipid analogue (32). Since we have found that the diffusion coefficients of Fab fragment–labeled class I MHC molecules and Fl-PE are identical in JY cell membranes, the diffusion of class I molecules under these conditions is unlikely to be constrained by cytoskeletal or other extramembranous interactions. The use of Fl-PE to probe cell membrane phospholipid mobility assumes that the lipid analogue does not itself perturb membrane organization. Interactions between, for example, the fluorescein moiety of the lipid probe and a more slowly diffusing membrane component could theoretically retard lateral mobility. If this were the case, then the lateral mobility of class I MHC proteins might not be controlled by the viscosity of the bilayer alone, but rather by constraints similar to those governing Fl-PE diffusion.

In peripheral blood lymphocytes (25), EBV-transformed B cell lines (31), murine lymphocytes (6, 13), and murine fibroblasts (8, 9) significant variation in class I MHC antigen mobility has been observed. A number of factors may influence the mobility of histocompatibility antigens. In both isolated plasma membranes (33) and intact fibroblast membranes (8), mobility is increased by treatment with either calcium ionophore or high phosphate buffer. Our data suggest that some of the variation may be explained by cell culture and cell labeling conditions. mAb microaggregates may also influence the mobility of class I MHC antigens. Although we did not observe clustering or patching of fluorescently labeled class I HLA molecules by fluorescence microscopy, it appears that microaggregates of mAb may induce a reduction in the mobility of these molecules on the surface of JY cells. This effect was observed with both intact mAb and Fab fragments, and was eliminated by high-speed centrifugation of the antibody. Oligomers but not monomers of IgE have recently been shown to induce a reduction in IgE receptor lateral mobility (20), and this reduction in protein mobility correlates with cell triggering and degranulation (19).

In EBV-transformed B cell membranes, class I MHC protein mobility is affected by both antibody valence and microaggregation. The same bivalent antibody probe used in the present study, W6/32-FITC, has been used to measure the lateral mobility of class I MHC proteins in membranes of interferon-γ–treated human dermal fibroblasts.[2] In these cells, neither the diffusion coefficient nor the fractional mobility of class I HLA molecules was dependent on the time of incubation with antibody. Further, both W6/32-FITC–labeled molecules and the lipid probe Fl-PE had fractional mobilities of 95–100% and diffusion coefficients of 1–5 × 10^{-9} cm^2 s^{-1} in human dermal fibroblast membranes.[2] Together with the present study, these observations suggest that cross-linking of class I MHC proteins induces constraints on the mobility of these molecules in B lymphoblastoid but not human dermal fibroblast cell membranes. Differential effects of W6/32-FITC on class I HLA molecule mobility may reflect differences in the biological responses of these two cell types to multivalent ligands for HLA molecules.

The extracellular substrate to which cultured endothelial cells are adherent affects the diffusion coefficient of plasma membrane lipid (23). In addition, the extracellular matrix and cell density affect the mobility of HLA antigens in adherent cultured fibroblasts (38). Here we report that cell density also influences the mobility of HLA antigens labeled with bivalent antibody in JY cells cultured in suspension. Cells grown in log phase or at high cell density were labeled with bivalent antibody and resuspended in identical solutions before lateral mobility measurements, eliminating the possibility that differences in fluorescent label concentration, phosphate concentration, pH, or ionic composition of the buffer contributed to the change in mobility, as has been observed in other systems (7, 8, 20, 33, 34, 39). There was no difference in MHC antigenic density, determined by fluorescence intensity measurements, between the cell populations. The absence of punctate fluorescence in cells grown either in log phase or at high cell density strongly suggests that W6/32-FITC–labeled class I MHC proteins were not spontaneously internalized into cytoplasmic vesicles. Consistent with this finding, others have reported that labeled class I MHC antigens are not internalized in either resting or activated B cells (35). Factors in addition to the extracellular substrate appear to alter the mobility of class I antigens. Such factors may include cell density–dependent changes in interactions between, for example, transmembrane class I molecules and cytoskeletal or membrane skeletal elements. Antigen cross-linking by bivalent antibody is required to induce immobilization of HLA antigens in cells grown to high cell density. The mechanism underlying this synergism between transmembrane protein cross-linking and growth to high cell density remains to be elucidated.

Specific interactions between class I MHC antigens and membrane hormone receptors, such as insulin receptors and epidermal growth factor receptors, have been demonstrated (10, 26, 29). These interactions suggest that MHC antigens may, under certain conditions, be distributed nonrandomly in the membrane. An association between MHC antigens and cytoskeletal proteins has been demonstrated by copurification of H-2 (15) and HLA-A2 (27) antigens with actin, but

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this association may be related to preparative conditions. A stable association between MHC antigens and cytoskeletal components has been difficult to document. MHC antigens are relatively resistant to capping (4) and do not associate with the detergent-insoluble cytoskeletal matrix as readily as surface immunoglobulin (5). Class I MHC antigens are not clustered in the plasma membrane as determined by the technique of fluorescence energy transfer (6). We have shown that the lateral mobility of MHC antigens labeled with purified, monovalent Fab fragments, which have been centrifuged to remove microaggregates, is identical to that of a lipid probe. Cross-linking of the surface molecules and growth to high cell density induce constraints on the lateral mobility of these transmembrane proteins.

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