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Immunology

Distribution and mobility of murine histocompatibility H-2K^k antigen in the cytoplasmic membrane

(rotational diffusion/translational diffusion/energy transfer/phosphorescence/flow cytometry)

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ABSTRACT **The topographical distributions and mobilities of the murine histocompatibility antigen H-2K^k and of concanavalin A (Con A) binding sites have been studied on a murine lymphoma cell line. The spatial distribution of H-2K^k antigens, the average distance between H-2K^k antigens and Con A binding sites, and the separation of different determinants on the H-2K^k antigen itself were determined by using fluorescence resonance energy-transfer measurements with a dual-laser flow sorter. From the lack of energy transfer between bound monoclonal anti-H-2K^k antibodies conjugated with fluorescein (donor) and rhodamine (acceptor), we conclude that the H-2K^k antigen exists without appreciable clustering on the cell surface. Substantial energy transfer between appropriately labeled Con A and antibodies bound to the H-2K^k antigen shows that the two populations are interspersed. Donor/acceptor pairs of monoclonal antibodies binding to different determinants on the same H-2K^k antigen exhibited a degree of energy transfer indicative of a mean separation of 8.6 nm between the sites. Time-resolved phosphorescence anisotropy measurements with anti-H-2K^k antibodies labeled with eosin or erythrosin yielded rotational mobility information for the antigen–antibody complexes on the cell membrane. The rotational correlation time of 10–20 μs and the finite residual anisotropy are compatible with an uniaxial mode of rotation of monomeric antigen around its transmembrane portion and, thus, provide additional evidence for an unclustered distribution. Capping by rabbit anti-mouse IgG immobilized the antigen–antibody complex. Fluorescence recovery after photobleaching was used to calculate an apparent lateral diffusion coefficient of $5 \pm 3 \times 10^{-10}$ cm^2·s^{-1} for the H-2K^k antigen labeled with fluoresceinated IgG or its corresponding Fab fragment.**

The *K* and *D* regions of the *H-2* major histocompatibility gene complex (MHC) of the mouse encode the class I antigens expressed on the surface of most somatic cells. The class I antigens consist of a highly polymorphic integral membrane glycoprotein of M_r 45,000 associated with an invariant β_2-microglobulin, a M_r 11,600 protein not coded for by the MHC. These antigens and other MHC gene products guide thymic lymphocytes in distinguishing self from nonself. The *H-2K* and *H-2D* regions provide the alloantigens that control (*i*) allograft rejection (1–3), (*ii*) induction of lytic (killer) T cells (1–4), and (*iii*) differentiation of restricted T cells that lyse cells bearing both a foreign (e.g., viral) antigen and a H-2K or H-2D antigen presented by the initial stimulating cells (4). Models for the mechanism by which the latter process occurs provide for the recognition of a complex between MHC products and foreign antigen on the target cell (altered self; refs. 2, 5–7) or both antigens separately (dual recognition; refs. 8 and 9). These models require a finite mobility and redistribution of the class I antigens on the cell surface. Considerations of a similar nature apply to other cell–cell interactions (10).

Although the primary sequences of the class I histocompatibility antigens are being determined (e.g., the *H-2K^b* haplotype; ref. 11), there have been few studies of their mobility (12, 13) or distribution in the plasma membrane, a prerequisite to the understanding of the dynamics of association and redistribution processes during T-cell function.

Site-specific monoclonal antibodies directed against the H-2K^k antigens have been made (14, 15) and provide a convenient method for labeling and monitoring the dynamics of the antigen–antibody complex on the cell surface. In addition, lymphoma cell lines with a homogeneous differentiation state have been selected for increased expression of the H-2K^k antigen of a single haplotype (16). Such cells (e.g., the LDBH T41 line used in the studies reported here) minimize the influence of heterogeneity in the differentiation or regulatory state of the cells on membrane composition and antigen distribution.

The plasma membrane is a complex structure of proteins, lipids, and underlying cytoskeletal elements, the dynamic interactions and distributions of which determine the functional state of the cell. A wide variety of physical–biochemical techniques have been used to study the proximity relationships and mobilities of the membrane components and their relationship to regulatory processes (17–30).

In our study of the murine histocompatibility H-2K^k antigen, we have selected techniques that may be applied to living cells: (*i*) flow cytometry for measuring fluorescence resonance energy transfer on a cell-by-cell basis—i.e., for probing proximity relationships on the cell surface (24–27); (*ii*) time-resolved polarized luminescence (22, 28, 29) associated with the triplet state (30) for studying the rotational mobility of membrane components; and (*iii*) fluorescence recovery after photobleaching (FRAP) for studying translational diffusion (21, 31).

MATERIALS AND METHODS

Cells. LDBH T41 (C3H × DBA/2) f1 cells (16) were grown in Dulbecco's minimal essential medium with 10% fetal calf

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Abbreviations: FRAP, fluorescence recovery after photobleaching; MHC, major histocompatibility complex; IgG, immunoglobulin G; Con A, Jack bean concanavalin A tetramer; FITC, fluorescein 5′-isothiocyanate; Me$_4$RITC, tetramethylrhodamine 5′-isothiocyanate; F-H-100 27/55, 30/6, and 5/28, fluorescein-labeled H-100 27/55, 30/6, and 5/28 antibodies; R-H-100 27/55, 30/6, and 5/28, rhodamine-labeled H-100 27/55, 30/6, and 5/28 antibodies; R-Con A, rhodamine-labeled Con A.

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serum and antibiotics. The viability of the cells after measurements was >90% as determined with fluorescein diacetate (32).

Labeled Monoclonal Antibodies and Concanavalin A (Con A). Monoclonal antibodies against H-2K^k antigens, H-100 5/28, H-100 27/55, and H-100 30/6, were purified from the culture medium of hybridoma cells (14) or from ascites fluid of BALB/c mice injected with the cells. (The cell lines were the gift of B. Holtkamp). A pure IgG protein was isolated by chromatography on protein A-Sepharose (Pharmacia). The H-100 5/28 and H-100 27/55 antibodies are directed against common determinants or sterically overlapping binding sites on the antigen, whereas the H-100 30/6 antibody is not inhibited in its binding by the other antibodies (14–16). All antibodies (IgG2 class) were conjugated with fluorescein 5′-isothiocyanate (FITC; Molecular Probes, Junction City, OR), tetramethylrhodamine 5′-isothiocyanate (Me_4RITC, Research Organics, Cleveland, OH), eosin 5′-isothiocyanate (Molecular Probes), or erythrosin 5′-isothiocyanate (gift from P. Garland) as described by Cherry *et al.* (33). (The FITC-conjugated antibodies are designated F-H-100 5/28, 27/55, and 30/6, whereas the Me_4RITC-conjugated antibodies are designated R-H-100 5/28, 27/55, and 30/6.) Jack bean Con A tetramer (Serva) was conjugated with FITC (F-Con A) or Me_4RITC (R-Con A) as described earlier (34). Labeled Fab fragments were obtained from H-100 27/55 antibodies after papain digestion and chromatography on protein A-Sepharose (35). The protein-to-dye labeling ratio was usually around 1:2 or 1:3. The activity of the conjugated antibodies was checked by competitive binding with unlabeled antibody (for FITC or Me_4RITC conjugates) or with fluorescein-labeled antibody (for eosin or erythrosin conjugates).

Specific Labeling of H-2K^k Antigens and Con A Receptors on the Cell Surface. Cell suspensions (100 μl; 10^7 cells ml^{-1}) in phosphate-buffered saline were incubated with a 5- to 10-fold excess of the labeled antibodies for 30 min at 37°C in the dark. The cells were washed and resuspended twice in buffer and used for measurements. To induce capping, a polyclonal rabbit anti-mouse IgG (Miles) was added, and the cells were incubated for 30 min at 37°C. Con A could be capped independently at the same temperature. To maintain Con A in a dispersed state, all labeling was carried out at 4°C, the distribution not being affected by the prior labeling or even capping of the H-2K^k antigen. To avoid temperature effects during the flow-sorter experiments, the cells were usually fixed after the labeling procedure with 1% HCHO for 30 min at 4°C and stored at 4°C prior to measurements. Data obtained with fixed cells did not differ from those of unfixed, viable ones.

Energy-Transfer Experiments. A flow sorter equipped with dual laser excitation was used to determine energy transfer between FITC- and Me_4RITC-labeled proteins bound to the cell surface. A complete technical description of the instrument and data analysis is given elsewhere (24, 25). With this method the energy-transfer efficiency (E) is evaluated for each cell, from which a frequency distribution for the population can be generated (25). E is directly related to the average donor–acceptor distance (36). Thus, the proximity of labeled antibodies binding the same H-2K^k antigen can be calculated, or, by using the analytic solutions to the energy transfer in two dimensions (37–40), an estimation of the average surface density of H-2 antigens and Con A binding sites may be made.

Measurements of Rotational Diffusion of H-2K^k Antigen by Time-Resolved Phosphorescence Anisotropy. The details of the instrument have been described (22, 28, 41). T41 cells were labeled with eosin or erythrosin H-100 27/55 whole antibody or Fab fragment. A dye laser at 515 nm driven by a pulsed N_2 laser was used as excitation source. The beam was focused on the middle of a (0.5 × 0.5 cm) fused-silica cell, containing 0.5 ml of 10^7 labeled T41 cells, purged free of O_2 with argon. Individual records were generated at 10 Hz; 4,096 records consisting of independent determinations of the parallel and perpendicular components of the gated phosphorescence emission were averaged. Corrections for the autoluminescence of the cells were made by taking records from unlabeled cells. Experimental phosphorescence decay curves were approximated by the simple expression (41):

$$r(t) = (r_{in} - r_\infty)\exp(-t/\phi) + r_\infty, \quad [1]$$

where Φ is the apparent rotational correlation time, r_{in} is the initial anisotropy, and r_∞ is the limiting anisotropy. Data were analyzed by multiexponential nonlinear regression techniques (42).

Measurements of Lateral Diffusion of F-H-100 27/55 and R-H-100 27/55 Antibody–Antigen Complexes. FRAP measurements of labeled H-100 27/55 antibodies attached to the plasma membrane of T-41 cells was carried out as described (43, 44). Individual cells suspended in phosphate-buffered saline, having an average diameter of 15 μm, were allowed to settle onto glass slides and were bleached with a Gaussian beam profile with a $1/e^2$ radius of 2.1 μm, using a Zeiss Neofluar 63/1.3 oil immersion objective. The Spectra Physics model 165 argon ion laser was operated at 200 mW (bleaching power; 10^{-4} of this value for the monitoring beam) with the 488-nm line for fluorescein-labeled cells and the 514-nm line for the rhodamine-labeled cells. The diffusion coefficients were calculated from the fluorescence recovery curves as described by Axelrod *et al.* (43).

RESULTS

Proximity Measurements Between H-2K^k Antigens and Con A Receptors. We investigated the distribution of the binding sites for Con A and the H-2K^k antigen on T41 cells by using energy-transfer measurements in a flow sorter. F-H-100 27/55 served as donor, and R-Con A served as acceptor. The cells were labeled sequentially with the donor concentration kept at a saturating level and with the acceptor concentration varied. Distributions of the resultant energy transfer calculated on a cell-by-cell basis showed that E increased with the acceptor concentration (Fig. 1).

Although the H-2K^k antigens and Con A binding sites exhibited energy transfer, indicating that they are in close prox-

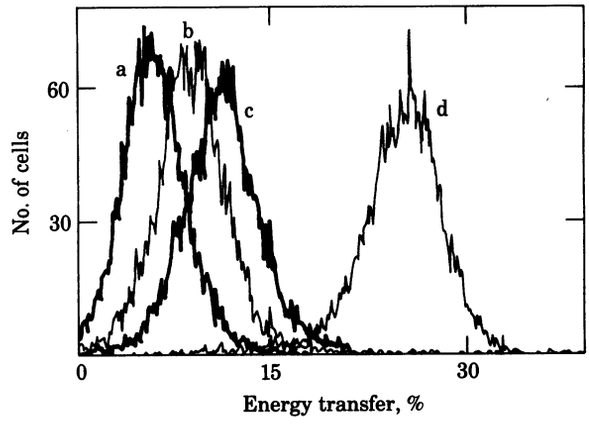

FIG. 1. Frequency distribution of energy-transfer efficiency between F-H-100 27/55 anti-H-2 antibody and R-Con A bound to T41 cell surface. During the labeling at 0°C, the antibody concentration was kept at saturation level, while the concentration of R-Con A was 12 μg/ml (trace a), 20 μg/ml (trace b), 30 μg/ml (trace c), and 200 μg/ml (trace d). Approximately 6,000 cells were analyzed per distribution.

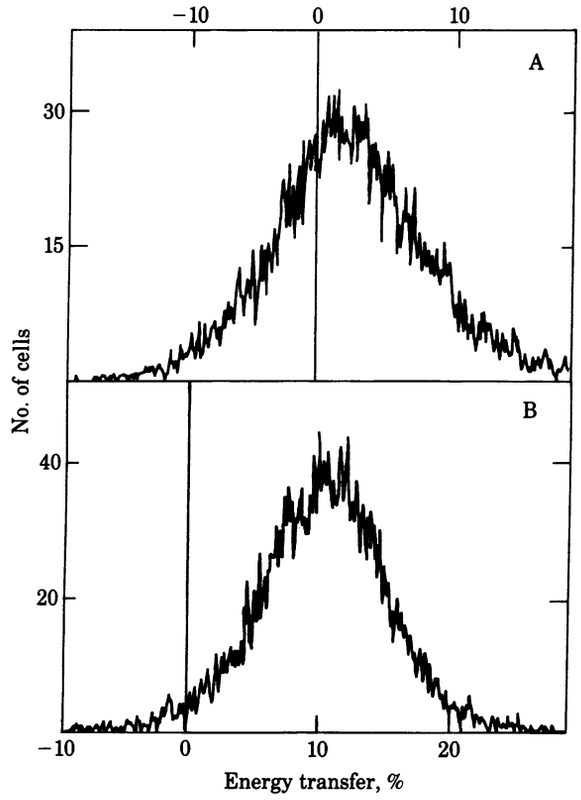

FIG. 2. Frequency distribution of energy-transfer efficiency between FITC- and Me_4RITC-conjugated noncompeting or identical monoclonal anti-H-2K^k antibodies bound to T41 cells. (*A*) F-H-100 27/55 and R-H-100 27/55 (identical) antibodies; the rhodamine-to-fluorescein molar ratio was 2. (*B*) F-H-100 27/55 and R-H-100 30/6 noncompeting antibodies. During the incubation the concentrations of antibodies were kept at saturation level. Approximately 6,000 cells were analyzed per distribution.

imity, we found no evidence for stable interactions. Capping of Con A binding sites by using R-Con A was followed by fluorescence microscopy and showed no redistribution of the disperse fluorescence pattern displayed by fluoresceinated anti-H-2K^k antibodies. Similar results were obtained by capping the H-2K^k antigen–antibody complex with rabbit anti-mouse IgG followed by binding of R-Con A at low temperatures. Independent and sequential capping of Con A and H-2K^k antigen–antibody complex resulted in an interspersed cap at the same pole of the cell.

Table 1. Proximity relationships between competing and noncompeting monoclonal anti-H-2K^k antibodies

Monoclonal antibodies	L, R/IgG	R'_0, nm	$\bar{E}$, %	$\mathcal{R}$, nm
Identical				
F-M1/R-M1	2.5	6.0	<3	>11
F-M2/R-M2	2.3	5.9	<3	>11
Noncompeting				
F-M1/R-M2	2.3	5.9	10.4	8.4
F-M3/R-M2	2.3	5.9	8.1	8.8

L, labeling ratio; $R'_0 = L^{1/6} R_0$ (36); R_0, critical transfer distance of isolated donor fluorescein (F) and the acceptor tetramethylrhodamine (R) chromophores at which the transfer efficiency is 50% [R_0, 5.1 nm for this dye pair (our determination; see also ref. 25)]; $\bar{E}$, mean value of the energy-transfer efficiency distribution histograms; $\mathcal{R}$, calculated separation distance; M1, M2, and M3, H-100 27/55, H-100 30/6, and H-100 5/28 anti-H-2 antibodies, respectively.

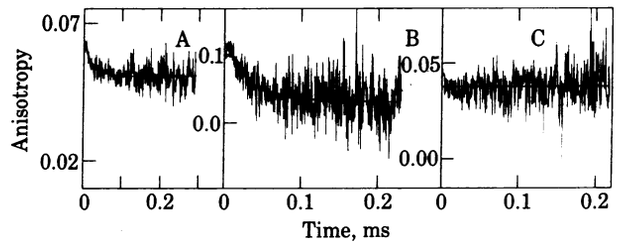

FIG. 3. Phosphorescence anisotropy decay kinetics of antibody–H-2K^k antigen complexes. The smooth lines represent the results of the computer fitting of the experimental curves to a single exponential decay (see also Table 2). T41 cells were labeled with H-100 27/55 anti-H-2K^k antibody conjugated to erythrosin (*A*) and the eosin-conjugated Fab fragment of the antibody (*B*). (*C*) Decay curve of eosin-conjugated H-100 27/55 antibody after the addition of polyclonal rabbit anti-mouse IgG under conditions inducing crosslinking—i.e., capping.

Because there is only one determinant on the H-2K^k antigen for a single monoclonal antibody, we were able to study the surface distribution of the antigens on the cells by using a mixture of the single-labeled derivatives F-H-100 27/55 and R-H-100 27/55 or F-H-100 30/6 and R-H-100 30/6 and measuring energy transfer in the flow sorter.

Both the H-100 27/55 donor/acceptor pair and the comparable H-100 30/6 pair gave energy-transfer distributions centered about zero for rhodamine/fluorescein molar ratios of 2:1 or 4:1 (see an example in Fig. 2*A*). Such distributions suggest that adjacent H-2K^k antigens are beyond the range of Förster-type resonance energy transfer (Table 1). The slight positive value of the energy-transfer mean was within the spread of values determined by an analysis of the statistical error function for the data (25).

Monoclonal antibodies that do not compete in binding to the H-2K^k antigen (14–16) can be used to determine distances between sites on the antigen itself. Three donor/acceptor pairs were constructed by using H-100 30/6 and either H-100 27/55 or H-100 5/28. The resultant energy transfer distribution for one pair is seen in Fig. 2*B*. Distances were calculated from the transfer efficiency (36) for all pairs and are given in Table 1.

Rotational Mobility of H-2K^k Antigen–Antibody Complexes. A suspension of T41 lymphoma cells labeled with eosin- or erythrosin-conjugated antibodies was used to measure the phosphorescence lifetime and anisotropy decay of the labeled cells. Fig. 3 shows the anisotropy decay curves of eosin- or erythrosin-labeled antigen–antibody complexes on the cell surface. The rotational correlation times were typically 10–20 μs at 25°C with either whole antibody or Fab fragment. The values of the correlation times as well as initial (r_{in}) and limiting (r_∞) anisotropy values are given in Table 2. A slight increase in the

Table 2. Summary of the phosphorescence anisotropy decay parameters for T41 cells labeled with eosin (Eo)- or erythrosin (Ery)-conjugated H-100 27/55 antibody (Ab)

Antibody	ϕ, μs	r_{in}	r_∞
Eo-Ab	17 ± 4	0.04 ± 0.01	0.028 ± 0.005
Ery-Ab	19 ± 5	0.05 ± 0.01	0.035 ± 0.005
Eo-Fab	22 ± 6	0.11 ± 0.01	0.033 ± 0.005
Eo alone	210 ± 20	0.13 ± 0.02	0.050 ± 0.010
Eo-Ab/RAMIG*	∞	0.04 ± 0.01	0.035 ± 0.007

The phosphorescence measurements were carried out at 25°C. Values are means ± approximate SD. Fab, Fab fragment of H-100 27/55 antibody; ϕ, correlation time; r_{in}, initial anisotropy; r_∞, limiting anisotropy.

* Capped by adding rabbit anti-mouse IgG (RAMIG) and measured at 37°C.

correlation time was observed when the anisotropy decay was measured at 4°C. Capping by addition of unlabeled Con A prior to labeling of cells with eosin-conjugated anti-H-2K^k antibody did not change the correlation times at 37°C or 4°C, showing that Con A capping does not redistribute (cluster) the H-2K^k antigen. A second unlabeled anti-H-2K^k antibody with specificity for a different determinant (H-100 30/6) did not change the observed correlation times either. However, dramatic changes were observed at 37°C when the eosin-conjugated H-100 27/55 antibodies were capped by adding rabbit anti-mouse IgG. The measured correlation times were 200 times longer than those of the uncapped samples, indicating an almost complete immobilization of the complex on this time scale.

The possibility that the phosphorescence signals are due to nonspecific labeling or autoluminescence of the cells was excluded by the following controls. The phosphorescence signals decreased as expected when unconjugated antibody was mixed with conjugated antibody for labeling (Fig. 4, curve b). A human lymphoma cell line, Namalva, showed negligible binding of erythrosin- or eosin-conjugated anti-H-2K^k (Fig. 4, curve c). Unconjugated eosin mixed with the cells gave a very long rotational correlation time (Table 2) and a phosphorescence intensity greater by an order of magnitude than that derived from the eosin-labeled antibody on the cells. Unlabeled cells showed autoluminescence; however, this signal had different decay and anisotropy patterns than those of the eosin- or erythrosin-labeled complexes (Fig. 4, curve d). We conclude that the calculated anisotropy decay is exclusively due to the rotation of the H-2K^k antigen–antibody complex.

Lateral Diffusion of the H-2K^k Antigen. FRAP experiments were made on T41 cells labeled with F- or R-H-100 27/55 antibodies. Fig. 5 shows the extent of the bleaching and the recovery kinetics. The apparent lateral diffusion coefficient at 22°C was $.5 \pm 3 \times 10^{-10}$ cm^2·s^{-1} (the average of five experiments) with the whole antibody or with Fab fragment of the R-H-100 27/55 bound to the cell surface. The extent of recovery varied in the range of 15–40%.

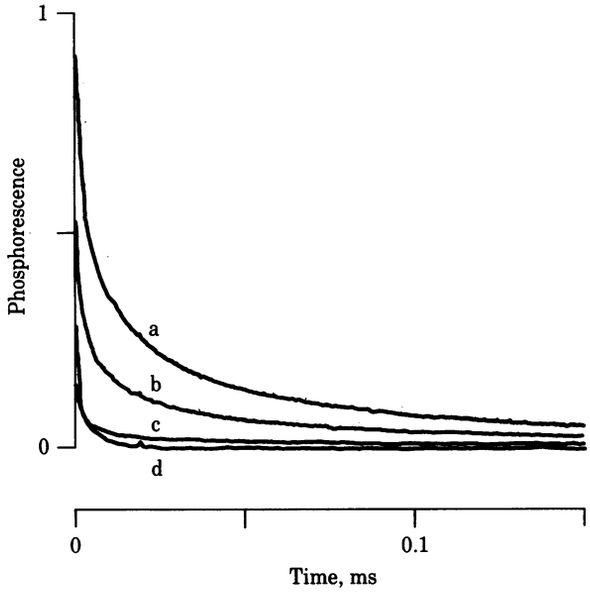

FIG. 4. Phosphorescence intensity decay of erythrosin-conjugated H-100 27/55 anti-H-2K^k antibody bound to cells. Curves: a, T41 cells labeled with erythrosin-conjugated antibody; b, T41 cells labeled with a mixture of erythrosin-conjugated and nonconjugated antibody (molar ratio, 1:3); c, human Namalva cells labeled with erythrosin-conjugated anti-H-2K^k antibody; and d, T41 cells labeled with nonconjugated antibody.

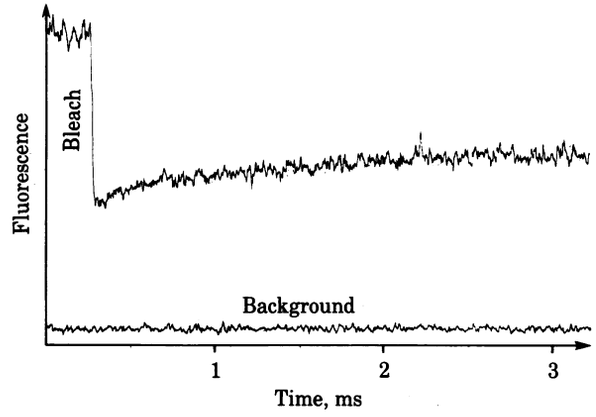

FIG. 5. FRAP curves of F-H-100 27/55 antibody bound to the surface of T41 cells. Cells were labeled with the conjugated ligands in the dark for 30 min at 37°C and measured at 22°C.

DISCUSSION

Amino acid sequence data are becoming available for H-2 antigen haplotypes (11). Models have been proposed for the tertiary structure of antigen–β_2-microglobulin complex and the manner of its insertion into the plasma membrane (11). A prerequisite to understanding how the H-2 antigens function is a detailed picture of their distribution and mobility on the cell surface under a variety of conditions and functional states. The only previous information regarding mobility and distribution was derived from lateral diffusion measurements with FRAP on the HLA and HLB antigens of human lymphoma cells (12) and the H-2 antigen on mouse fibroblasts (13). Our data agree with these two studies in showing a diffusion coefficient not different from that of most integral membrane proteins (21) and a low fractional recovery. The latter finding has been attributed variously to the involvement of the cytoskeleton in direct or indirect contacts with the integral protein being studied or to the distribution of the antigen in domains that do not freely exchange on the cell surface during the time scale of the experiment. It is important for the evaluation of models concerning H-2 function to determine whether dimer or higher-order clusters of H-2 exist in the plasma membrane and whether they exhibit rotational freedom.

Earlier data with polyclonal anti-H-2 antisera alone showed that the antigens cap poorly. Even with an additional second antibody, they cap slowly compared to surface IgG (45). Thus, a highly clustered distribution of the H-2 antigen is not expected. The T-200 glycoprotein on mouse thymocytes has been shown to cocap with H-2K antigens (5). However, we have observed no interaction between Con A binding glycoproteins and the antigens, as evidenced by their ability to cap independently. Other measurements of Con A binding sites alone had indicated that these proteins are not distributed evenly on the surface of T41 cells (25). The high energy transfer we observed in the present study between anti-H-2K^k antibodies and Con A even at low occupancy of the lectin sites suggests that the most likely distribution is one in which the H-2 antigens are dispersed randomly throughout the Con A domains. Lateral diffusion and cocapping studies by Cartwright *et al.* (46) on the H-2K^k antigen and vesicular stomatitis virus glycoprotein G in reconstituted membranes show no stable association of the two proteins.

The existence of clustering of the H-2K^k antigens seems unlikely by the absence of significant energy transfer when the cells were incubated with a mixture of fluorescein- and rhodamine-labeled antibodies of the same monoclonal type. Smith

and co-workers (12) in fluorescence microscope studies of lymphoma cells labeled with two fluorophores conjugated to the same monoclonal antibody observed macroscopic capped distributions. Although these authors concluded that the antigens are monomeric and disperse, such data can be ambiguous as has been demonstrated amply by data on peptide hormone receptors (47, 48), which display a uniform distribution in the fluorescence microscope but are microaggregated in clusters of up to 50 receptors by rotational relaxation measurements (47).

Our rotational relaxation measurements on the H-2K^k antigen yielded correlation times consistent with the picture of a monomeric protein embedded in the plasma membrane, freely rotating about its membrane portion. The correlation times are comparable to those obtained for other integral proteins—e.g., the acetylcholine receptor under certain conditions (49) and the epidermal growth factor receptor (47). The non-zero r_∞ is strong evidence for hindered rotation (22, 30). The low initial anisotropy r_{in} we attributed to local motion of the phosphorescent probe and to the segmental motions of the antibody and the externally disposed regions of the H-2 antigen to which it is complexed. Such processes taking place in the aqueous medium would be expected to occur in the submicrosecond time domain (22, 30). The observed phosphorescence intensities were similar to those obtained in other studies of hormone receptors comparable in number to the H-2K^k antigen (47). Further evidence for specificity was given by competitive binding experiments and by use of cells lacking the H-2 antigen. Neither Con A binding and capping nor addition of a second monoclonal anti-H-K^k antibody significantly affected the rotational freedom of the antigen, providing further evidence that homologous or heterologous associations are not significant. However, addition of a crosslinking ligand such as rabbit anti-mouse IgG immobilized the antigen.

In conclusion, a combination of biophysical measurements of H-2K^k antigen on living cells gives a consistent picture of a monomeric, freely rotating, dispersed antigen, close to but not associated with Con A binding glycoproteins. However, its lateral mobility is restricted, possibly because of interactions with underlying cytoskeletal elements. This picture provides the basis for further studies concerning the relationships between H-2 antigens and viral antigens in complex reactions such as cytotoxic T-cell lysis.

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