DIFFUSION RATES OF CELL SURFACE ANTIGENS OF MOUSE-HUMAN HETEROKARYONS

I. Analysis of the Population

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ABSTRACT

The rate of appearance, in a newly formed heterokaryon population, of cells bearing completely intermixed mouse and human surface antigens may be used to estimate diffusion constants for antigens on individual cells. From this estimate, it appears that the surface antigens in most cells do not diffuse at the rate expected, but rather move more slowly, by a factor of ten or more, than expected from either measured or calculated diffusion constants for proteins freely mobile in the plane of a lipid membrane. Differences in diffusion rates between cells are not due to effects of Sendai virus, or of trypsin. Restrictions on diffusion are apparently not due to cytochalasin B- or Colcemid-sensitive elements.

KEY WORDS diffusion · cell surface heterokaryons · surface antigens · Sendai virus

Previous study of the arrangement of cell surface antigens in heterokaryons has indicated that some of these antigens, integral membrane proteins, are mobile in the plane of the membrane, and that their rapid intermixing, upon formation of a heterokaryon, is due to diffusion rather than to cell metabolic activity (12). While the study of membrane antigens of heterokaryons has been further used to make qualitative or comparative examinations of rates of protein lateral diffusion (9), little attention has been paid to quantitative aspects of the system. In the present paper, we present some more quantitative aspects of the interdiffusion of surface antigens in heterokaryons, together with some data on restrictions on their diffusion.

MATERIALS AND METHODS

Reagents and Solutions

HEPES-buffered Hanks' balanced salt solution was prepared without $NaHCO_3$ or phenol red, and with 0.01 M HEPES buffer (*N*-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid). Phosphate-buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 3.7 mM Na_2HPO_4. Cell-dispersing solution, CTC, consisted of 2.5% heat-inactivated chicken serum, 0.2% crystalline trypsin, and 0.002% collagenase (Worthington Biochemical Corp., Freehold, N. J.) in calcium- and magnesium-free Hanks' solution. For some experiments, cells were dispersed with 1 mM disodium ethylenediamine-tetraacetic acid in PBS adjusted to pH 7.35. Paraformaldehyde powder (Matheson, Coleman, and Bell, Cincinnati, Ohio) was used to prepare fixative solutions by dissolving the powder in hot (80°C) PBS to final concentrations of 0.1–2.0%. Freshly prepared solutions at these concentrations do not induce autofluorescence in fixed cells. Cytochalasin B (Aldrich Chemical Co., Miluaukee, Wis.) was made up as a 3 mg/ml stock in dimethyl sulfoxide (DMSO) and diluted into HEPES-Hanks' solution for use. Colcemid was made up as a 10 μg/ml stock in PBS.

Cell Lines

Mouse C1 1d cell line, a TK^- variant of LM cells (4), was maintained in Eagle's minimal essential medium (MEM) (Grand Island Biological Co., Grand Island,

New York) with 5% fetal calf serum, and without antibiotics; WI-18-VA-2, an 8-azaguanine-resistant SV40-transformed human cell line (20), was similarly cultured. SA-I sarcoma cells (5) were carried as an ascites tumor in strain A/J mice.

Marker Antigens and Antibodies

The mouse cell line C1 1d, derived from the CBA strain, continues to express mouse major histocompatibility antigens, H-2 antigens. The human cell line displays at least one equivalent human antigen, HLA-A2. Mouse H-2 antigens were detected with a (C57BL/6 × DBA/2)F1 B10.Br antiserum, as previously described (8), followed by a fluorescein-conjugated goat anti-mouse Fab reagent (12). Human HLA-A2 antigens were detected with an alloantiserum "Stoltzfus" kindly provided by Dr. Wilma Bias of the Department of Medicine, Johns Hopkins Medical School (Baltimore, Md.). Visualization of bound anti-HLA antibody was achieved with a tetramethylrhodamine-conjugated goat anti-human IgG (Baltimore Biological Laboratories, Baltimore, Md.) (1).

Antibody Specificity

Neither fluorescent conjugate bound to either of the cells used, whether or not these cells had been first treated with Sendai virus. However, both the anti-H-2 reagent and the anti-HLA reagent reacted with inappropriate cells treated with Sendai virus at the concentrations used in our fusion experiments (see below). The HLA reagent also reacted with untreated mouse cells. These inappropriate reactivities were removed by absorbing Stoltzfus with 1×10^7 C1 1d/ml of undiluted serum for 30 min at 4°C, followed by absorption of 1 vol of serum with 1 vol of packed SA-I cells. Anti-Sendai virus activity was removed from both anti-H-2 and anti-HLA antisera by incubating the sera with 300–600 HAU of UV-inactivated Sendai virus for 30 min at 37°C, followed by overnight incubation at 4°C. Virus and any remaining cell fragments were cleared by centrifuging the absorbed sera at 20,000 *g* for 30 min. The absorbed sera gave only expected reactions with Sendai-virus-treated cells: Stoltzfus reacted only with VA-2 and not with C1 1d cells, while the anti-H-2 reagent reacted only with C1 1d and not with human, VA-2, cells.

Sendai virus was prepared as described (3), but was inactivated with B-propriolactone. The virus preparation was the generous gift of Dr. Hayden Coon.

Cell Fusion and Formation of Heterokaryons

In a standard experiment, C1 1d and VA-2 cells were harvested by exposure to CTC for 2–3 min, washed twice with HEPES-Hanks' solution, and combined in ratios of C1 1d/VA-2 = 2:1. A maximum of 3×10^6 cells were resuspended in 0.1 ml of 300–400 HAU/ml Sendai virus in HEPES-Hanks' solution and agitated at 0°–4°C for 10 min. The suspension was then removed to 37°C and shaken for 3 min to induce fusion. After this brief incubation, the virus fusion factor was inactivated by dilution with 10% fetal calf serum. The sparse cell suspension in Hanks' solution, with relatively few heterokaryons, was further incubated at 37°C. At intervals, aliquots of the heterokaryon mixture were removed to ice, until completion of sampling. The antigens were visualized by simultaneous treatment with 50 μl of anti-H-2 reagent and 50 μl of anti-HLA reagent for 15 min at 0°–4°C. Antiserum was diluted with cold HEPES-Hanks' solution to which 2.5 M dinitrophenol (DNP) was added to inhibit pinocytosis. After two washes, the cells were simultaneously reacted with 50 μl each of fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-human antibodies, for 15 min at 0°–4°C. Conjugate was diluted with cold HEPES-Hanks, and the cells were washed twice to remove all free dye. Cells were normally scored live, but when fixed were treated with 0.2–0.5% paraformaldehyde in HEPES-Hanks' solution for 1 h at 0°–4°C. The cells were gently washed in HEPES-Hanks' solution and resuspended in HEPES-Hanks' solution with 5% fetal calf serum, before examination by fluorescence microscopy.

Cytochalasin B or Colcemid Pretreatment of Parent Cells

C1 1d and VA-2 cells were trypsinized, washed twice with HEPES-Hanks solution and separately resuspended in 1 ml of HEPES-Hanks containing 0.3, 3.0, or 30 μg of cytochalasin B or 10 μg of Colcemid. Control cells for cytochalasin B experiments were suspended in 1% DMSO. After 30-min incubation at 0°–4°C, C1 1d and VA-2 cells were mixed and fused in the usual way.

Cell Size Measurements

C1 1d and VA-2 cells were fused under standard conditions. The diameters of cells from 0 and 40-min samples were then measured at 400×, using a calibrated eyepiece micrometer.

Surface Antigens in Fixed Heterokaryons

Fusion was initiated as described above, but after 3 min at 37°C the cells were pelleted in a refrigerated centrifuge and resuspended in chilled 0.2% paraformaldehyde in PBS. Cells were fixed for 1 h in ice. They were then washed with HEPES-Hanks' solution and returned to 37°C. Samples of the fixed population were stained in the usual manner.

Microscopy

Fluorescent heterokaryons were scored with a Leitz Ortholux microscope equipped with a fluorescence vertical illuminator after Ploem. The interference filters and mirrors supplied with the illuminator were used for excit-

ing fluorescein or tetramethylrhodamine. With this system, a 40 × numerical aperture 1.3 objective could be used at full aperture, giving images of exceptional quality and brightness.

ANALYSIS OF THE DATA

As described in detail in Materials and Methods, the H-2 and HLA antigens of mouse-human heterokaryons are visualized with multiple antibodies applied to samples of the heterokaryon population taken at intervals after initiating fusion. Thus, data on antigen mobility in the plane of the membrane can be presented only in terms of the frequency in the total cell population of heterokaryons with a particular arrangement of mouse and human antigens. The technique, then, does not allow a time history of a single heterokaryon, but rather gives only the time history of a cell population, with respect to the frequency of particular cell types in that population.

After staining, cells are classified either as having completely intermixed their surface antigens, or as having still segregate antigens, with portions of the membrane staining only for mouse or human antigens. Examples of intermixed and segregate cells are given in a previous paper (12). In that paper the term "mosaic" was used for a cell that appeared to have completely intermixed surface antigens. In the present paper, we emphasize restriction on mobility of the antigens, and data are plotted in terms of the fraction of all heterokaryons remaining segregate at a given time.

RESULTS

Results averaged from seven experiments are plotted in Fig. 1. Loss of segregates from the population, an indication of the rate of diffusion of membrane antigens, follows a first-order decay curve at least for 20 min after first sampling the heterokaryon population. Intermixing of antigens of fixed heterokaryons (filled circles of Fig. 1) follows a similar decay curve. It should also be noted that the first sample is taken for staining 3 min after initiating fusion. Heterokaryons in this sample may have had up to 2.5 min or more time to rearrange their surfaces before sampling. As can be seen in Fig. 1, the first sample in fact contains around 10% of cells that appear to have completely intermixed their surfaces in the 3 min before sampling.

The remaining heterokaryons convert from segregate to intermixed at slower rates. This conversion appears to occur through intermediates. If, instead of scoring cells only as segregate or intermixed, a more careful determination is made to find cells with partly intermixed surface antigens, it is found that, at the level of resolution available, even at the earliest time of scoring, most of the

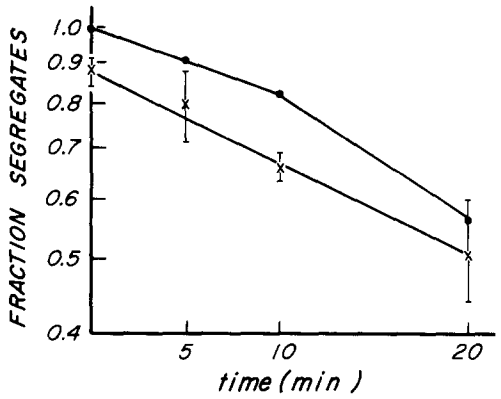

FIGURE 1 Loss of segregate cells, bearing separate membrane areas of mouse H-2 and human HLA antigens, from a population of heterokaryons. The first sample was taken 3 min after initiating heterokaryon formation. All times are taken relative to the initial sample. (×) Mean of seven control experiments. The bars show the range of values found for each time point. (●) Values for a single experiment in which cells were fixed in paraformaldehyde solution after heterokaryon formation but before further incubation at 37°C.

apparent segregates in fact have partly intermixed their antigens (Fig. 2). By 23 min after initiating fusion, no cells remain as true segregates; all cells that have not completely intermixed their surface antigens are at least part way through the process. Thus, the heterokaryon population displays a range, possibly a continuum of diffusion rates for a given set of antigens on different cells.

In two experiments, cells were fused with varying concentrations of Sendai virus (Table I). Though fusion efficiency varied with virus dose, the rate of conversion of segregate cells to intermixed did not. The rate of conversion of segregates also was not affected if cells were removed from plates with EDTA instead of CTC (Fig. 3) or if CTC treatment was prolonged to 10 min (Fig. 4). In other experiments (data not shown), heterokaryons were formed from cells removed from plates with CTC and allowed to heal at 37°C for 1 h or 18 h. The heterokaryon populations formed from healed cells lost segregates at the same rate as those formed from freshly removed controls.

The distribution of diffusion rates was not altered by treating the parent cells of heterokaryons with a high concentration of the tubulin-dissociating drug, Colcemid (Fig. 5). Treatment of the cells with cytochalasin B reduced the mobility of surface antigens in a substantial fraction of the population (Fig. 6).

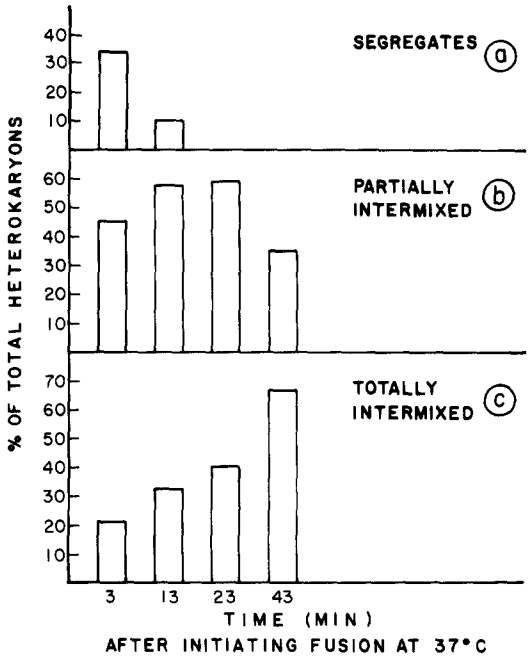

FIGURE 2 Percentage of all heterokaryons with surface antigens. (*a*) Segregate, (*b*) partly intermixed, and (*c*) fully intermixed. Times here are taken from the point of initiation of fusion.

TABLE I

Effect of Sendai Virus Dose on Cell Fusion and on Diffusion Rate of Surface Antigens

Sendai virus concn	Cells with two or more nuclei	Time after initiation of fusion (min)		
		3	8	23
HAU/ml	%		*% Segregates*	
1,500	30	88	79	62
750	20	91	77	61
300	13	86	76	60
150	12	87	81	63
75	7	89	70	62
35	8	86	83	56

The data presented are from two experiments. In one, cells were fused with varying concentrations of virus and sampled only 23 min after initiating fusion. This sample was also used to determine the percent of cells with two or more nuclei, a measure of fusion efficiency that includes both hetero- and homokaryons. In the second experiment, antigen distribution was determined at 3 and 8 min, and no measurement was made of fusion efficiency.

400-500 cells were counted for the fusion efficiency measurements. 12-100 heterokaryons were scored for antigen distribution in each tube. The number scored was always more than 50 for tubes of the higher virus concentrations (150-1,500 HAU/ml).

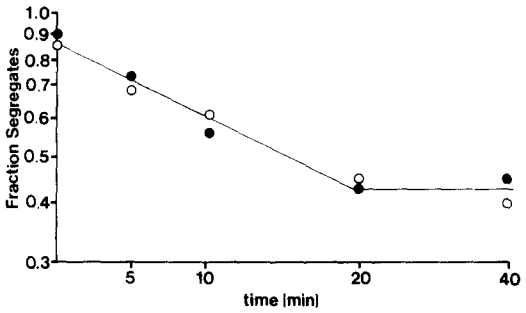

FIGURE 3 Comparison of heterokaryons formed from cells removed from culture plates with CTC (○), or with EDTA (●).

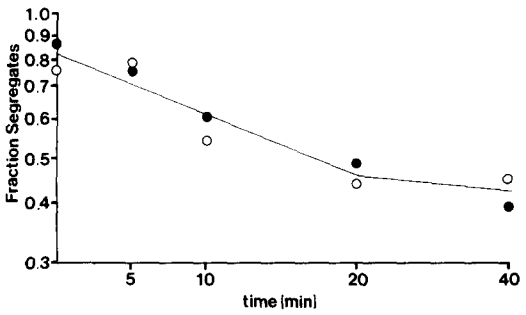

FIGURE 4 Comparison of heterokaryons formed from cells removed from plates by treatment with CTC for 2-3 min (○) with those exposed to CTC for 10 min (●).

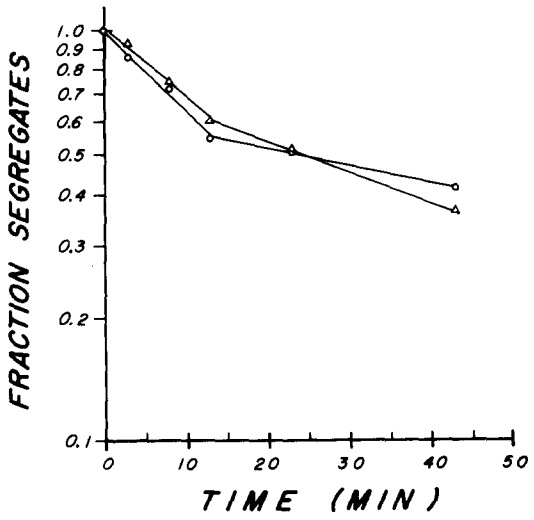

FIGURE 5 Effect of Colcemid treatment of parent cells on the rate of interdiffusion of surface antigens in heterokaryons. (○) Untreated control; and (△) parent cells treated with 10 μg/ml Colcemid before fusion.

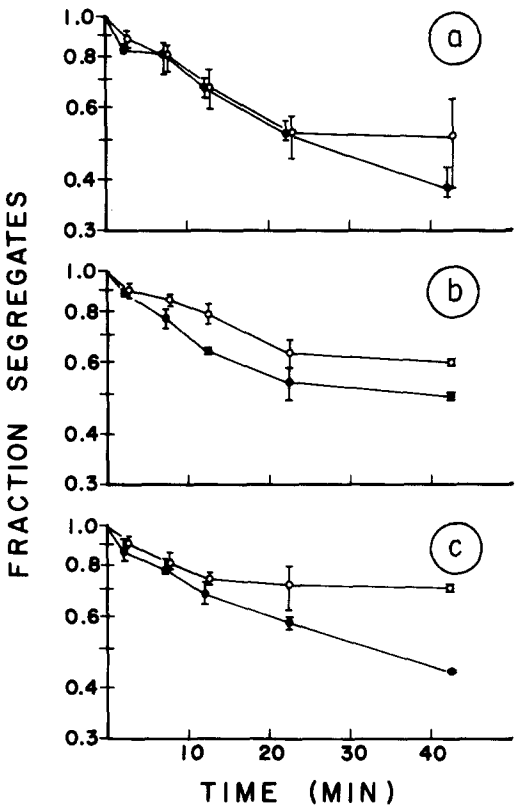

FIGURE 6 Effect of cytochalasin B treatment of parent cells on the interdiffusion of surface antigens in heterokaryons. (●) Control cells pretreated with 1% DMSO; (○) cells treated with cytochalasin B. (*a*) 0.3 μg/ml cytochalasin B; (*b*) 3.0 μg/ml cytochalasin B; and (*c*) 30 μg/ml cytochalasin B. Three experiments were done at each of the drug concentrations indicated and the data averaged. Only some of these experiments were taken to 40 min after fusion, and hence data points for these times are without error bars in Fig. 6*b* and *c*.

DISCUSSION

A population of mouse-human heterokaryons contains cells with two basic distributions of H-2 and HLA surface antigens, segregate or intermixed. With time, the population changes from mainly segregate heterokaryons to mainly intermixed heterokaryons. This change occurs even in fixed cells, reinforcing our belief that the redistribution of heterokaryon antigens is due to diffusion, and not to some active cellular process.

Huang (13) has solved the equation for the diffusion of particles from the surface of one hemisphere to the other of a sphere. Our heterokaryons approximate spheres; Sendai virus-treated cells including those used by us are poor in microvilli (reference 14; S. O. Rosenberg and M. Edidin, unpublished observations). Huang derived an expression for the half-time of interdiffusion in the case described, $\tau = r_0^2/2D$, where r_0 is the radius of the sphere, and D is the particle diffusion constant. Measurement of 54 variously-treated binucleate heterokaryons gives us an average value of 21 μm diameter with a range of 20–27 μm. The diffusion constant for the antigens that we use as markers is expected to be 1–4 × 10^{-9} cm^2 s^{-1} based on the best published value for protein lateral diffusion in membranes (20). A diffusion constant was calculated from (see reference 11)

$$D = \frac{KT}{6\pi a\eta}.$$

With values of membrane lipid viscosity of $\eta = 1$ P (summary of probable values for lipid viscosity in reference 7) and particle radius, a, about 40 Å, an expected value of about 4 × 10^{-9} cm^2 s^{-1} was yielded. These diffusion constants substituted in Huang's equation predicted that, at a maximum, interdiffusion ought to appear to be complete in 2–8 min after formation of a heterokaryon. At this time, the concentration of antigen per unit area would be about 45% of its concentration in the parent cells. Judging from experience, we can detect antigens in concentrations lower than this though not at 1/10 this concentration. Therefore, antigen interdiffusion would appear complete within 1–4 min after fusion. Only a small proportion of heterokaryons appear with intermixed antigens at the expected times. These comprise 10–15% of the first population sample taken 3 min after initiating fusion. The remaining heterokaryons do not interdiffuse their surface antigens for some time longer than the predicted time.

The population heterogeneity observed does not appear to be due to differential effects of trypsin and collagenase on individual cells. Prolonged treatment with CTC of parent cells does not change the distribution of segregates vs. time in the heterokaryon population formed from such parents. Also, no differences were seen when comparing heterokaryons formed from cells released from plates by EDTA with those formed from cells released by a routine brief treatment with CTC.

Sendai virus-induced fusion is known to alter the membrane permeability of Ehrlich ascites cells (17), and indeed any fusing agent is expected to alter cell membrane structure. However, neither the fraction of cells with intermixed surface anti-

gens found in our earliest time samples nor the proportion of segregate cells 23 min after initiating fusion was affected by the amount of Sendai virus used for fusion, over a 40-fold range of virus, though the fraction of multinucleate cells fell fourfold over this range of virus. With published values as a guide, we have calculated the distribution of the number of virus particles per cell, using a Poisson distribution. Only 1% of the cells exposed to 75 HAU/ml of virus will bear as many particles as the mean number of particles on cells exposed to our standard concentration of virus, 300 HAU/ml.

Two alternate explanations may be offered for the population heterogeneity observed, and for the first-order decay curve plotted in Fig. 1 for fraction of segregate heterokaryons persisting with time. First, the antigens of most heterokaryons may be locked in place, and unable to diffuse until some event occurs at random, at some time after heterokaryon formation. After such an "unlocking" event, the marker antigens would interdiffuse in 2–8 min. An alternative explanation of the data of Fig. 1 is that most heterokaryons slow the diffusion of their surface antigens to some extent. A continuum of rates of diffusion is seen in the population, with half of the cells showing apparent diffusion of antigens at least 10 times slower than predicted for free diffusion. If the first explanation, random rapid release of an absolute anchor, is correct, then few intermediate arrangements of antigens should be detectable. If the second explanation is correct, then many cells that are scored as segregate, or at least with surface antigens not yet fully intermixed, should, on close inspection, prove to bear some areas in which mouse and human surface antigens have intermixed, and other areas in which antigens of only one species are displayed and which have not yet been reached by the antigens of the second species. Such cells are seen; indeed, cells with partly interdiffused surface antigens form a majority of these cells conventionally scored as segregate. Hence, we interpret the curve shown in Fig. 1 as the time history of a population containing cells whose membrane-integral H-2 and HLA antigens are not completely free to diffuse on the cell surface. Of course, this observation is also consistent with a relatively slow unlocking process occurring at random. Diffusion in the membrane of any given cell would begin as soon as such slow unlocking began and would increase in rate when unlocking was complete.

From Huang's equation and the average diameter of heterokaryons, we estimate that antigens on heterokaryons that appear intermixed only after 20 min have an apparent diffusion constant, $D = 2 \times 10^{-10}$ cm^2 s^{-1}. Similar values have been measured at 25°–30°C, by another technique, photobleaching and recovery of fluorescence not requiring virus treatment of cells, as the average for generally labeled surface membrane proteins of C1 Id cells (10) and of L^{-6} rat myoblasts (24). Thus, the median apparent diffusion constant for heterokaryon surface antigens and the average diffusion constant for generally labeled surface proteins accord well (lateral diffusion is relatively insensitive to mol wt differences, varying only as $\sqrt[3]{\text{mol wt}}$).

It should be noted that the maximum diffusion constant predicted, and apparently found in heterokaryons, was not seen in experiments on generally labeled proteins. It may be then that Sendai virus treatment does alter the membranes studied, to some extent, although this may simply be due to a slight swelling effect which causes loss of many microvilli (14), hence making the actual surface area of the cells more closely approximate the area calculable from cell diameters measured in the light microscope. Estimates of microvilli on L-cells (15) and on some cells of other cultured cell lines (2) indicate that the actual area may be from three to sevenfold greater than that calculated from cell diameter measurements. This difference could account for the failure, with photobleaching techniques, to observe diffusion of heterokaryon antigens at a rate as high as that seen in other experimental systems (21). Another possibility, not controlled for in published work on photobleaching, is that laser damage to the cell membrane slows diffusion of marker proteins. Hence, it is not clear whether the small fraction of heterokaryons with freely diffusing antigens is due to the Sendai virus, or whether the failure to detect such cells by photobleaching is due to an artifact of that method.

The population distribution of diffusion rates for H-2 and HLA antigens reported here is only weakly a function of the cell cycle. In a series of experiments on heterokaryons formed from synchronized cells, it has been found that cells in all stages of the cycle yield about the same fraction of heterokaryons allowing rapid or intermediate rates of diffusion of their surface antigens. Cells in S phase yield a somewhat greater proportion of heterokaryons with greatly restricted mobility of surface antigens than those in other parts of the cycle (19).

Judging from experiments in which Colcemid

and cytochalasin B were used, the restriction of free diffusion is not due to anchoring to microtubules or microfilaments, such as has been found in lymphocyte capping and other systems (6, 18, 23, 28, 29). Indeed, addition of cytochalasin B appears to slow diffusion of antigens in some cells, a phenomenon also reported in another system for measuring lateral diffusion in membranes (24).

Data on the distribution of antigens in heterokaryons may be used to infer a range of diffusion constants for membrane H-2 and HLA antigens on single cells, though the data themselves give a time history of the sorts of cells to be found in the heterokaryon population. The antigens observed in our experiments are held in the membrane in a manner similar to that of other membrane integral proteins (16, 25), for example, erythrocyte membrane glycoproteins (27), cytochrome b_5 reductase (26), and possibly vertebrate rhodopsin (22). We suggest that their diffusion behavior may be generalized to that of other membrane integral proteins.

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