

# Lateral Diffusion of H-2 Antigens on Mouse Fibroblasts

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**ABSTRACT** We have used fluorescence photobleaching and recovery (FPR) to measure the lateral diffusion of mouse H-2 antigens, labeled with fluorescent Fab fragments, in the membrane of c1 1d fibroblasts. Diffusion coefficients,  $D$ , vary more than 20-fold from cell to cell, though they vary no more than twofold when measured at different points on a single cell. The fraction of H-2 antigens mobile,  $R$ , also varies from cell to cell, and no lateral diffusion of H-2 antigens can be detected in ~20% of the cells examined.

Treatment of cells with NaCN + NaF, reducing their levels of ATP reduces the proportion of cells in which no lateral diffusion can be detected. The maximum values of  $D$  seen in poisoned cells are less than those in controls. Treatment of cells with the divalent ionophore, A23187, greatly increases the proportion of cells in which diffusion of H-2 is rapid,  $D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ .

The data obtained on diffusion by FPR can be replotted in the form of an experiment in which lateral diffusion of H-2 antigens is measured in a population of heterokaryons. There is good agreement between this transformation and actual data on heterokaryons. Thus the two methods appear to measure the same transport process.

The lateral diffusion of membrane integral proteins was first demonstrated in a population of heterokaryons formed from mouse and human parent cells (10). Quantitative analysis of such populations yields estimates of the diffusion coefficients,  $D$ , for mouse and human MHC antigens, H-2 and HLA, which vary by more than 20-fold within a population of heterokaryons (6). The proportion of heterokaryons allowing diffusion of MHC antigens at high rates,  $D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ , is increased if heterokaryon parent cells are depolarized with ouabain or are treated with the divalent ionophore A23187 in the presence of external  $\text{Ca}^{++}$  (7, 8).

Fluorescence photobleaching recovery, FPR, is another technique for measuring lateral diffusion of membrane proteins. The technique may be applied to single cells, unmodified by virus. Here we show that FPR measurements of the lateral diffusion of MHC antigens in one of the cell lines previously studied in heterokaryons yield a wide range of diffusion coefficients,  $D$ . The distribution of  $D$  well approximates the distribution estimated for the antigens in heterokaryons. The ionophore, A23187, shifts the distribution of  $D$  in the population of single cells to higher values, as it does in heterokaryons. Sodium cyanide and NaF also appear to modify constraints to lateral diffusion.

## MATERIALS AND METHODS

Mouse c1 1d and human VA-2 cells were grown on glass in Eagle's medium supplemented with 5% fetal calf serum (Reheis, Phoenix, Arizona) MEM-5, in a 5%  $\text{CO}_2$ /air atmosphere. Cells were removed from plates with chicken

serum:trypsin:collagenase (2.5%, 0.2%, 0.002%) CTC. The divalent ionophore A23187 (18) was the gift of Dr. Robert Hammill, Lilly Research Laboratories (Indianapolis, IN). It was made up as a 100 $\times$  stock in dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO). NaCN and NaF were Baker reagent grade (J. T. Baker Chemical Co., Phillipsburg, NJ).

In some experiments cells were fused with Sendai virus; samples of the heterokaryon population were taken at intervals after initiating fusion H-2 and stained with TMR Fab anti-H-2 or fluorescein Fab rabbit anti-WI-38. Diffusion of antigens in these cells was compared with diffusion of H-2 antigens that were labeled on c1 1d before fusion with VA-2. Details of the fusion and staining procedure are given in earlier papers (6, 8).

11-4.1 hybridoma cells, producing an IgG2a against previously undescribed public antigen of H-2<sup>k</sup> (15) were grown in MEM + 10% fetal calf serum. Spent culture medium was stored at  $-20^\circ\text{C}$  until needed. A liter or more of culture medium was brought to pH 8.0 with NaOH and then slowly applied to a 3.5-ml protein A-Sepharose (Pharmacia Inc., Piscataway, NJ) column. After loading (usually overnight) and washing the column in pH 8.0, 0.1 M phosphate buffer until no O.D. 280 was detected in the eluate, the column was eluted with pH 4.5, 0.1 M citrate buffer. Collecting tubes contained sufficient solid Tris base to neutralize the buffer (9). Yields of protein were 10–20 mg per liter of medium. Both pH 8 and pH 4.5 eluates were checked for reactivity with c1 1d by indirect immunofluorescence. pH 8 eluates that stained c1 1d were again passed over protein A-Sepharose.

The eluted IgG was concentrated to 5–10 mg protein/ml by dialysis against solid polyvinyl pyrrolidone (GAF) and dialyzed against digestion buffer, 0.15 M NaCl, 0.1 M potassium phosphate, 0.001 M  $\text{Na}_2$  EDTA, pH 8.0. The 4 ml of concentrated solution contained ~25 mg of protein. 80 U of 2 $\times$  crystallized papain (Sigma Chemical Co., 20 U/mg) were activated for 15 min at  $25^\circ\text{C}$  in 0.1 M sodium acetate, 0.01 M cysteine, and 0.001 M EDTA buffer, pH 5.2. 40 U of activated papain were added to the protein solution, which was then incubated at  $37^\circ\text{C}$  for 10 min. The digestion was stopped by adding 40 mg of iodoacetamide to the solution (final concentration 0.05 M) (11). The digested protein was passed over a 200-ml column of Sephadex G-100 in Dulbecco's phosphate-buffered saline (PBS). Four peaks were found in all preparations, though their proportions

varied from digestion to digestion. Peak 2 with apparent molecular weight 45,000 is Fab; most mouse Fc is digested to smaller fragments. This was confirmed by passage of peak 2 over protein A Sepharose. Most of the O.D. 280 passed through the column at pH 8.0. Peaks 1, 2, and 3 all stained c1 1d in indirect immunofluorescence. Rechromatography of each peak on G-100 showed that the bulk of the activity was in peak 2. Yields of peak 2 ranged from 25–50% of theoretical.

The 9–12 O.D. of peak 2 were concentrated to 1.5 ml and dialyzed against 0.5 M carbonate buffer, pH 9.5. The Fab was conjugated with tetramethylrhodamine isothiocyanate (Bioquest, Cockeysville, MD) for 2½ h at 25°C. The solution was passed over Sephadex G-25 in PBS to separate free dye from conjugate. It was necessary to further dialyze the conjugate against a suspension of mixed bed ion exchange resin (Amberlite MB-3) in PBS in order to remove traces of free dye. If this was not done cells, though ring stained, also stained internally. Samples of conjugate that had been stored for more than a few weeks were routinely passed over G-25 immediately before staining cells.

The conjugate had an average dye:protein ratio of 1.0. It was stored at –20°C at a concentration of 1.5 mg/ml in PBS.

We have since used the procedure described above to conjugate other IgG2 monoclonal antibodies (12, 16). The yield of Fab fragments varies as does the titer of the final conjugate. We also find that conjugates can be most efficiently purified and freed of excess dye by passage over DEAE-Sephadex or DEAE cellulose in pH 7.4, 0.01 M phosphate buffer. The most lightly coupled Fab fail to bind to the column whereas more heavily conjugated fractions may be eluted with 0.05 and 0.1 M buffer steps in a manner similar to that described for intact Ig (5).

c1 1d were labeled for FPR by mixing  $3 \times 10^5$  cells in 0.1 ml of Hanks' BSS (buffered with HEPES to pH 7.3 and 1% in newborn calf serum) with 1 volume of diluted conjugate, for 15 min at 0°C. Excess conjugate was washed away in Hanks' BSS. The cells were taken up in rectangular glass capillary tubes (Vitro Dynamics, Rockaway, NJ) and checked for ring stain before FPR measurements. Cells treated with A23187 (20 µM final concentration in Hanks' BSS; 1.2 mM in Ca<sup>++</sup>) or NaF + NaCN (each 1 mM in Hanks' BSS) were washed with the solutions of the compounds after staining and were held at 0°C in Hanks' BSS containing the compounds for no longer than 30 min. Cells were washed free of excess A23187 before making FPR measurements, but cells treated with NaF + NaCN were not washed.

Our FPR apparatus has been described in detail elsewhere (20). It is based on a Control Laser 2.5-W argon-krypton laser (Control Laser Corp., Holobeam Laser, Inc., Orlando, FL) and a Leitz Ortholux II fluorescence microscope, fitted with a  $\times 40$  oil immersion objective n.a. 1.30. The laser beam was split, and one beam attenuated, as suggested by Barisas and Leuther (3), but using optical flats instead of mirrors (14). Emitted light was detected by an EMI 978A photomultiplier tube, and a PAR 1140A quantum photometer. Data was recorded on a Hewlett-Packard 320 dual channel DC amplifier recorder. For our experiments we used the 531-nm laser line focused to an exp (–2) radius of  $1.2 \pm 0.2$  µm. Monitoring intensities were 3 µW, and bleaching times were 20 ms. Under these conditions localized heating of the membrane should be less than a few tenths of a °C (1).

The fluorescence from a spot on the labeled membrane is monitored by the attenuated laser beam. To bleach, light intensity is briefly increased  $10^{-3}$  to  $10^{-4}$  fold typically bleaching 80–90% of the fluorescence. Light is then returned to monitoring levels and the recovery of fluorescence due to diffusion of unbleached molecules into the spot observed. Two parameters are obtained from FPR measurements, the fraction of dye molecules free to diffuse,  $R$ , and the half-time,  $t_{1/2}$ , for recovery of fluorescence from which one obtains the diffusion coefficient,  $D = \gamma w^2 / 4t_{1/2}$ , where  $w$  is the  $1/e^2$  beam radius and  $\gamma$  is a coefficient dependent on the degree of bleaching (2).

Rebleaching of a given spot (after increasing the gain of the chart recorder) usually gave a  $t_{1/2}$  close to that of the initial measurement but with a greater  $R$ . This tactic was used to make accurate measurements of  $D$  in cells in which recovery after the initial bleach was low. The values for  $D$ , but not those for  $R$ , from second bleaches are included in the data.

All measurements were made at 30°C using a Bailey Instruments (Saddle Brook, NJ) temperature-controlled stage that was calibrated to determine the actual temperature at the point of observation when the oil immersion lens is in thermal contact with the slide. Each measurement required 1–5 min. Measurements were made only on rounded cells. No capillary was examined for more than 60 min. Metabolically driven flows are common in c1 1d kept at 30°C; recordings with shapes characteristic of flow (2) were not analyzed.

## RESULTS

We produced active Fab fragments from a monoclonal mouse alloantibody by following a modified procedure for papain digestion in which the enzyme is activated before addition to the substrate and in which digestion times are kept short (11).

The TMR-conjugated Fab fragments bound specifically to mouse L cells, c1 1d. Cells treated with 5–50 µg/ml of conjugated protein showed bright ring fluorescence. The binding of the conjugated fragment was blocked by excess unlabeled antibody. However, once bound the fragment could not be eluted off labeled cells by excess antibody. Thus binding appears to be specific and sufficiently stable so that the recovery of fluorescence observed after bleaching is not due to desorption and resorption of labeled Fab. The Fab fragments do not themselves interfere with lateral diffusion of H-2 antigens.  $D$  for H-2 inferred from timed samples of mouse-human heterokaryons was the same whether the H-2 antigens were labeled in the parent c1 1d or in the heterokaryons (Fig. 1).

Average values of our FPR measurements are given in Table I. The average diffusion coefficient,  $D$ , is significantly greater ( $p < .001$ ) for A23187 cells than for control or NaCN + NaF-treated cells. In turn, the average diffusion coefficient of H-2 in control cells is nearly twice that in metabolically poisoned cells ( $p < .01$ ). It may also be seen that the standard deviation for NaCN + NaF-treated cells is only around ¼ that of the other two groups. The average fraction mobile,  $R$ , of labeled H-2 is similar for control and NaCN + NaF-treated cells.  $R$  for A23187-treated cells is significantly less than  $R$  for control cells ( $p < .001$ ).

There was no correlation between  $D$  and  $R$  in a sample of each population tested for such correlation. Also,  $D$  and  $R$  did not correlate with the time after bringing the cells to 37°C at which a measurement was made. The proportion of control cells showing extreme values (high or low) of  $D$  and  $R$  varies from experiment to experiment. Such variation has also been seen in our experiments with heterokaryons.

The average values of  $D$  in Table I are distorted by the omission of very low values in cells scored as not showing any recovery of fluorescence during an experiment (between 10 and 20% of all cells). A clearer evaluation of  $D + R$  is given by their distributions shown in Figs. 2 and 3. Metabolic poisons

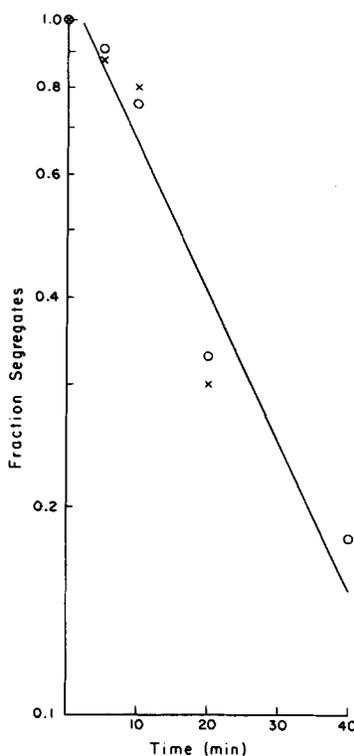


FIGURE 1 Lateral diffusion of H-2 antigens in mouse-human heterokaryons. Tetramethylrhodamine Fab anti-H-2 (TMR Fab) was bound to cells either before (x) or after (o) fusion.

TABLE I

Average Lateral Diffusion Coefficients and Fractional Mobilities of Fab-labeled H-2 Antigens on Mouse cl 1d Fibroblasts

Treatment	n*	D‡	SD	n	R	SD
—	276	1.6	1.6	335	31	34
NaCN + NaF	203	0.9	0.42	218	33	21
A23187§	262	2.5	1.7	235	23	15

\* Number of cells in the group.

‡  $\times 10^9 \text{ cm}^2$ . Values are averaged only for cells showing some measurable recovery,  $D > 2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$ . NaCN + NaF,  $0.75 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ , A23187  $1.8 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ .

§ Some cells were treated with NaCN and NaF as well with A23187.  $D$  and  $R$  for these cells were no different from average values for cells treated with A23187 alone and hence they are included in the total.

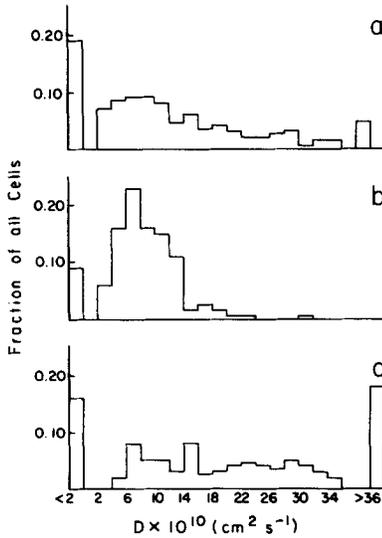


FIGURE 2 Distribution of diffusion coefficients,  $D$ , in (a), control cl 1d; (b), cl 1d treated with NaCN plus NaF and (c), cl 1d treated with A23187.

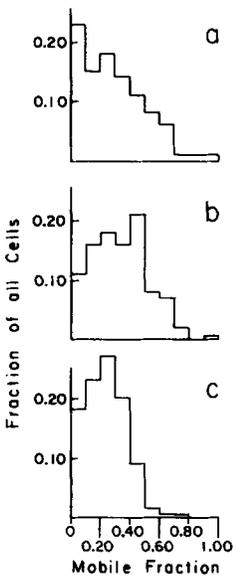


FIGURE 3 Distribution of the fractional mobility,  $R$ , in (a), controls; (b), NaCN plus NaF-treated and (c), A23187-treated cl 1d.

reduce the range of  $D$  in the treated cell populations (Fig. 2b) compared to controls (Fig. 2a). Few NaCN + NaF-treated cells restrict diffusion to  $< 2 \times 10^{10} \text{ cm}^2 \text{ s}^{-1}$  compared to controls, and very few poisoned cells show  $D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$  (Table II). The distribution of  $R$  in the poisoned cells (Fig. 3b) approximates that of the controls (Fig. 3a). However,  $R$  is very low in 11% of poisoned cells, compared with 23% of controls (Table II). The proportion of A23187-treated cells in which  $D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$  is over twice that in control (Fig.

3c).  $R$  for A23187-treated cells averages  $\frac{1}{3}$  of that in control cells and only 2% of ionophoretreated cells have more than 50% of labeled antigens mobile (Table II).

$D$  and  $R$  for control and A23187-treated cells was not altered by fixation in a poorly cross-linking fixative, 5% paraformaldehyde, for 30 min at  $0^\circ\text{C}$ . Diffusion was blocked (10/13 cells showed no recovery) when Fab-labeled cells were treated with divalent anti-Ig, a cross-linking antibody. Values of  $D$  at different points on the same cell differed at most by twofold (Table III).

In a single series, 33 cells, Sendai virus treatment did not significantly alter either the average diffusion coefficient,  $D = 1.37 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$  or its standard deviation  $\pm 1.06 \times 10^{-9}$ , from that of controls.

We have previously measured lateral diffusion of H-2 antigens in populations of mouse-human heterokaryons. In Fig. 4 we plot  $Y$ , the fraction of all cells in which  $D$  measured by FPR is greater than some value, against an "effective time," the calculated time for MHC antigens to diffuse from one hemisphere to another of a cell the size of a heterokaryon at the specified values of  $Y$ . This is equivalent to plotting the fraction of cells in a heterokaryon experiment remaining segregate at a given time after initiating fusion (see for example Fig. 1). The data for control and A23187 treated cells reproduce fairly well the first 13 min of a heterokaryon experiment, though the proportion of cells in which H-2 antigens diffuse slowly,  $D < 5 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$  (equivalent to times  $> 13$  min) is higher than expected.

## DISCUSSION

The original demonstration of lateral diffusion of membrane proteins (10) was based on the analysis of timed samples of a population of newly formed mouse-human heterokaryons. Diffusion coefficients for H-2 antigens of individual cells are inferred from the known diameter and calculated surface area of the heterokaryons and from the appearance of the cells at the time of sampling (13). Heterokaryons whose surface antigens have intermixed must allow diffusion at a rate sufficient to achieve intermixing of the two marker antigens by the time

TABLE II

The Distribution of Diffusion Coefficients and Fractional Mobilities Measured in Various Treated Populations of cl 1d Fibroblasts

Treatment	Percent of Cells with			
	$D < 2 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$	$D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$	$R < 0.1$	$R > 0.5$
—	19	22	23	17
NaCN + NaF	9	1.5	11	18
A23187	16	48	18	2

TABLE III

Lateral Diffusion of H-2 Antigens in Two or More Places on a Single Cell

Cell	$D_1$	$D_2$	$D_3$
1	12	24	—
2	6.2	7.7	—
3	5.9	6.1	4.9
4	6.9	8.3	5.9
5	2.2	2.9	—
6	11	8.8	—
7	5.9	4.6	—

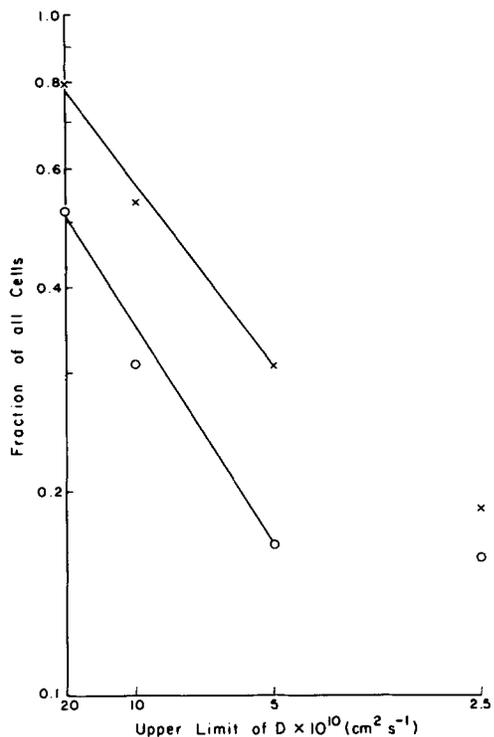


FIGURE 4 The data on distribution of diffusion coefficients present as they would appear in a population of heterokaryons for control ( $\times$ ) and A23187-treated cells ( $\text{O}$ ).

of sampling (6, 13). From this analysis we concluded that most heterokaryons restrict diffusion of their H-2 (and HLA) antigens to some extent.

Using FPR, a single-cell technique, we have reinvestigated lateral diffusion of H-2 glycoproteins in the mouse cell, c1 1d, used in heterokaryon experiments. We found that the distribution of diffusion coefficients among single c1 1d followed the distribution estimated for heterokaryons. A fraction,  $\sim 20\%$ , of the controls showed  $D > 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ , a value that would result in heterokaryons with intermixed surface antigens in the earliest samples of a newly fused population. This compares with 0–20% of cells with intermixed surface antigens actually observed in heterokaryon populations 3 min after initiating cell fusion. (Fig. 1 and references 6 and 8). The range of variation found in the population of cells is greater than that found when lateral diffusion is measured at several sites on a single cell. Because it is also found in populations of heterokaryons induced by Sendai virus, it seems that there is an intrinsic cell-to-cell variation in constraints to lateral diffusion of H-2 antigens that is not affected by Sendai virus and that is relatively invariant from place to place over a single cell. Of course, the second cell population in the fusion, VA-2, must also contain some cells in which diffusion is unrestricted. We do not know the effect of fusion of a cell in which  $D$  is unrestricted with a cell in which it is restricted.

The median  $D$  for all control cells,  $9 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$  is somewhat faster than the  $4 \times 10^{10} \text{ cm}^2 \text{ s}^{-1}$  calculated for the same H-2 antigen label in heterokaryons (Fig. 1). Given the assumptions involved in the calculation (notably that of the minimum amount of antigen diffusing from a source hemisphere that can be detected in a sink hemisphere) and the factors of added virus and human cell membranes we feel that this is good agreement. Perhaps more important than the quantitative comparison is the fact that the population of single

cells has a distribution that extends from values expected for viscosity-limited diffusion to values more than 10-fold slower. A similar large range of diffusion coefficients for HLA antigens on normal lymphocytes and on EBV-transformed cells has been shown by pattern photobleaching (17, 19). In those experiments a large fraction of cells ( $\sim 50\%$  of lymphocytes) showed no recovery of fluorescence in bleached spots.

The changes in the distribution of  $D$  and  $R$  after treatment with metabolic poisons appear to involve a shift of some cells from a condition in which lateral diffusion of all H-2 antigens is very slow or totally inhibited to a condition in which at least some H-2 molecules on the surface of a given cell are free to diffuse in the time of the experiment. However, the poisons also appear to reduce lateral diffusion rates of H-2 antigens in some cells, because the number of cells in this group in which  $D > 1.4 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$  is greatly reduced. Thus both extremes of the distribution of  $D$  are reduced by NaCN + NaF.

A23187-treated cells gave a distribution of  $D$  that well approximated the distribution inferred from heterokaryon experiments. A23187 appears to have several different effects on lateral diffusion of MHC antigens. The most striking effect is the increase in diffusion coefficient of MHC antigens in many cells to values  $> 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ . This increase apparently comes at the expense of cells in which  $2 \times 10^{-10} < D < 2 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ ; the ionophore does not seem to affect cells in which lateral diffusion of MHC antigens is highly restricted. A23187 also reduces the mobile fraction of MHC antigens on many cells.

The results with metabolic poisons and A23187 suggest that there may be two different sorts of restraints to lateral diffusion of MHC antigens, as is the case in erythrocytes (10a). One restraint would immobilize all the antigens on a cell for the time scale of our experiment. This restraint seems sensitive to NaCN plus NaF (and see reference 4). A second restraint would modulate the lateral diffusion rate of antigens that are mobile to some extent. A23187 appears primarily to affect this second class of restraint. Further work is required before this suggestion of two sorts of restraints to lateral diffusion, can be verified.

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