# Effects of Cell Density and Extracellular Matrix on the Lateral Diffusion of Major Histocompatibility Antigens in Cultured Fibroblasts

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*Abstract.* We have studied the effect of cell density on the lateral diffusion of major histocompatibility (MHC) antigens in the plasma membranes of fibroblasts using fluorescence recovery after photobleaching. The percent recovery of fluorescence was decreased in fibroblasts grown in confluent cultures. While recovery of fluorescence was measurable in >90% of the cells from sparse cultures, measurable recovery was detected in only 60–80% of the cells from dense cultures; no mobile antigens were detectable in 20–40% of cells examined. The diffusion coefficient on human skin fibroblast cells that did show recovery was the same for cells grown in sparse or dense conditions. In WI-38, VA-2, and cl 1d cultures the diffusion coefficients of mobile antigens were smaller in cells from dense cultures. Changes in lateral diffusion occurred with increased cell–cell contact and with age of cell culture but were not observed in growth-arrested cells or in sparse cells cultured in medium conditioned by confluent cells. Decreased mobile fractions of MHC antigens were observed when cells were plated on extracellular matrix materials derived from confluent cultures. Treatment of the extracellular matrix materials with a combination of proteolytic enzymes or by enzymes that degrade proteoglycans abolished this effect. Matrices produced by cells from other cell lines were less effective in inducing changes in mobile fractions and purified matrix components alone did not induce changes in lateral diffusion. Finally, there were no differences in the proportion of MHC antigens that were resistant to Triton X-100 extraction in sparse and dense cells. These results suggest that cell–cell interactions mediated through extracellular matrix materials can influence the lateral diffusion of at least part of the population of MHC antigens.

LATERAL and rotational motions of proteins within the plane of the membrane have been implicated in the control of many cellular processes, including signal transduction (2, 9, 28) and cell–cell interactions (10, 15). The lateral diffusion of many cell surface proteins has been measured on cultured cells using a variety of techniques (1, 2, 21, 30, 38). These studies have emphasized that the lateral motions of most membrane proteins are highly constrained. Diffusion coefficients for membrane proteins are generally 10–100 times slower than those measured for lipid probes in the same cells or than predicted from considerations of molecular size and hydrodynamic theory (52). In studies using fluorescence recovery after photobleaching, the recovery of fluorescence is often incomplete and in some cases no recovery can be measured, suggesting that the protein is immobile on the time scale of the experiment (16, 30). Proteins such as acetylcholine receptors at the neuromuscular junction (3), sodium channels on skeletal muscle cells (49), and lectin receptors on polarized epithelia are highly immobile (14, 60).

The mechanisms constraining the lateral diffusion of membrane proteins are not well understood. While the cytoskeleton may restrain lateral diffusion of some molecules such as band III in the erythrocyte (24, 48) and acetylcholine receptors at the neuromuscular junction (54), other membrane proteins appear to be subject to different types of constraints (1, 26, 38).

The lateral diffusion of the major histocompatibility (MHC)[1] antigens has been studied extensively in fibroblasts and lymphocytes (11, 16, 25, 42, 58). The class I MHC antigens consist of a membrane-spanning heavy chain of $M_r$ 44,000 with a cytoplasmic tail of 31–46 amino acids and an associated light chain of $M_r$ 12,000. On fibroblasts, diffusion coefficients varied from <1 to 20 × $10^{-10}$ $cm^2/s$ within the population but varied by less than twofold at different places on the same cell (16). The recovery of the signal in the photobleached area was usually not complete and a percentage of the cells showed no measurable recovery. Similar results were obtained in fluorescence photobleaching studies of MHC antigens on lymphocytes (11, 25, 42).

In this paper, we examine the effects of cell culture conditions on the lateral mobility of the MHC antigens. We show

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; HSF, human skin fibroblasts; MHC, major histocompatibility complex.

that fibroblasts cultured at high cell density exhibit reduced mobile fractions and present data suggesting that extracellular matrix (ECM) materials elaborated by dense cells can affect the lateral mobility of MHC antigens. We also show that the changes in antigen mobility between sparse and dense cells are not correlated with increased association of these antigens with Triton X-100 cytoskeletons.

## Materials and Methods

### Cells and Cell Culture

Mouse cl 1d and human VA-2 cells were maintained in Eagle's minimum essential medium supplemented with 5% fetal calf serum. WI-38 were grown in minimum essential medium supplemented with 10% fetal calf serum and normal human skin fibroblasts (HSF) (ATTC #CRL 1508, 3-day male) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fresh stocks of WI-38 and HSF were thawed every 6–8 wk and cells were used before growth rates decreased. Cells were removed from tissue culture dishes and passed with chicken serum/crystalline trypsin/collagenase (2.5:0.2:0.002%). In some experiments, cells were plated onto glass coverslips. Sparse cultures were <1 × 10³ cells/cm², whereas dense cultures ranged from 3 to 5 × 10⁵ cells/cm² and showed maximal cell contact.

### Monoclonal Antibodies, Preparation of Fab, and Conjugation Procedures

Human cells were labeled for fluorescence photobleaching with tetramethylrhodamine- or fluorescein-isothiocyanate conjugates of Fab fragments of the monoclonal antibody, KE-2. This antibody reacts with all class I HLA gene products and was a gift of Dr. Roger Kennett, Department of Genetics, University of Pennsylvania. Mouse cells were stained with rhodamine- or fluorescein-conjugated Fab fragments of monoclonal antibody 11-4-1. Preparation of the Fab fragments, conjugation procedures, and cell staining methods were as described elsewhere (16). In some cases, the rhodamine-conjugated antibodies were further purified using SM-2 beads using a method to be described in more detail elsewhere. Briefly, SM-2 beads were swollen in methanol, then rinsed in distilled water and phosphate-buffered saline (PBS). Immediately before use, rhodamine-conjugated antibody was incubated with SM-2 beads (25 μg of wet beads/100 μl of antibody at final dilution) for 15 min at 4°C.

### Fluorescence Photobleaching and Recovery, and Data Analysis

Lateral diffusion of antibody-labeled MHC antigens was measured by spot fluorescence photobleaching and recovery. Our experimental apparatus was described in detail in a recent paper (57). Briefly, a small spot on the cell membrane is bleached by an intense pulse of a focused laser beam several milliseconds in duration, then the laser beam is attenuated and used to monitor the recovery of fluorescence in the same spot. The instrument has been modified so that the beam used is chopped by a fast shutter to reduce problems of bleaching of fluorescence by the monitoring beam. Data are collected and analyzed by an IBM PC computer using custom designed hardware and software. Two parameters are obtained from each curve: the half-time, tau, from which the diffusion coefficient can be calculated, and the percent recovery. Measurements were made at 18–20°C except as noted.

Analysis of a large number of diffusion coefficients from similar cultures suggested that the diffusion coefficients from fibroblasts have a lognormal rather than a normal distribution. Statistical comparisons were performed using a logarithmic transformation of the diffusion coefficients and histograms display these transforms. The recovery values were compared using the Mann-Whitney-Wilcoxon nonparametric U test (46). Cells showing no recovery were included as a zero in the average of the recoveries but were not included in calculations of the average diffusion coefficients.

### Preparation of ECM

ECM was prepared by a modification of a previously described method (45) from cells maintained under confluent conditions for at least 7 d. Cells were removed from the coverslips using 0.01 M EDTA in PBS containing 1 mM phenylmethylsulfonyl fluoride. The dishes were washed three times with minimum essential medium/10% fetal calf serum and examined for residual cells. Fresh cells were plated onto these coverslips under sparse conditions.

ECM-coated plates were treated with various combinations of trypsin (0.2%; Worthington Biochemical Corp., Freehold, NJ; twice crystallized), collagenase (0.02%; Worthington Biochemical Corp.), and chicken serum (2.5%) for 15 min at 37°C, then washed several times in media. In some experiments, chromatographically purified collagenase was used (Form III, Advance Biofactures Corporation) and was reconstituted at 2,000 U/ml in 0.025 M Tris, 0.01 M calcium acetate, pH 7.4. For digestion of proteoglycans, plates were exposed to hyaluronidase (Sigma Chemical Co., St. Louis, MO; crude, 0.4% in acetate buffer, pH 6.0) or chondroitinase ABC (4 U/ml; in 10 mM Tris-acetate, 10 mM NaCl, 0.02% bovine serum albumin) for 2 h at 37°C. Heparan sulfates were digested by treating plates with nitrous acid at room temperature for 90 min.

Types I and III collagen were solubilized in 0.5 M acetic acid at a concentration of 1 mg/ml. Solutions were diluted to 10 μg/ml in distilled water, then dried onto the dishes and sterilized under ultraviolet light in a laminar flow hood. Laminin or fibronectin (100 μg/ml) were either coated onto glass coverslips or were added to the media (10 μg/ml final concentration). Heparin (mucosal; Calbiochem-Behring Corp., La Jolla, CA), chondroitin sulfates (A-whale cartilage, B-porcine skin), and hyaluronic acid (human umbilical cord; Miles Laboratories Inc., Elkhart, IN) were made up as stock solutions (100 μg/ml) and added to the media containing cells at the time of plating at a final concentration of 10 μg/ml.

### Triton X-100 Cytoskeletons

KE-2 antibody was labeled with ¹²⁵I using the chloramine T method. Cells were plated under sparse or dense conditions in 24-well plates. Cultures were incubated with 1 × 10⁻⁸ M antibody, which represented 60,000 cpm, in PBS containing 0.1% bovine serum albumin at 22°C for 2 h. Drugs were added to the cells during this 2-h incubation. Cells were washed twice to remove excess antibody, then incubated for 10 min in either PBS or a 17-mM phosphate buffer, pH 6.1 containing 1 mM EDTA and 1% Triton X-100 (23). The supernatant was counted and the insoluble material was removed by incubation for 15 min in 0.1 N NaOH. The counts were normalized to the counts remaining after incubation with PBS only.

## Results

### Lateral Diffusion of MHC Antigens Measured on Sparse and Confluent Cells

Table I summarizes fluorescence photobleaching and recovery measurements on sparse (1–3 × 10³ cells/cm²) or dense (3–5 × 10⁵ cells/cm²) cultures of fibroblasts. Cells grown in confluent conditions showed a decrease in the average percent recovery compared to cells from sparse cultures. This decrease was due both to an increase in the number of cells
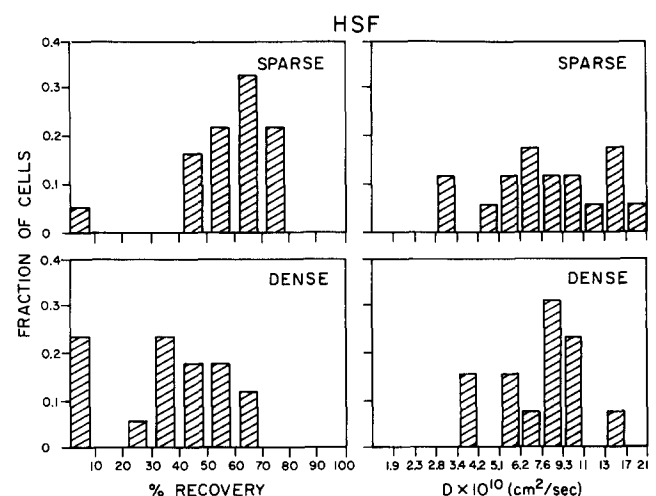


Figure 1. Distributions of percent recoveries (mobile fractions) and diffusion coefficients for MHC antigens on sparse and dense HSF cells labeled with KE-2 antibody. Diffusion coefficients are plotted on a log scale.

**Table I. Effects of Cell Density on Lateral Diffusion of MHC Antigens**

| Cell type | Culture conditions | Recovery | | Diffusion coefficient of mobile antigens ($\times 10^{10}$ cm$^2$/s) | |
| | | Average % recovery | Cells with no recovery | Mean | 95% C.I. |
|---|---|---|---|---|---|
| **A. Measurements made at 19°C** | | | | | |
| HSF | Sparse | 58 (4.2)* | 1/18 (6)‡ | 8.0 | 6.1–8.3 |
| | Confluent | 29 (5.5)§ | 8/21 (38) | 7.5 | 6.0–9.4 |
| WI-38 | Sparse | 36 (8.0) | 0/20 (0) | 9.0 | 7.3–11.1 |
| | Confluent | 24 (3.7)§ | 12/29 (41) | 4.7¶ | 3.1–7.3 |
| VA-2 | Sparse | 32 (2.8) | 1/18 (6) | 6.5 | 4.7–9.0 |
| | Confluent | 25 (3.7) | 9/33 (27) | 4.9¶ | 3.9–5.9 |
| cl 1d | Sparse | 47 (6.4) | 1/14 (7) | 7.5 | 5.0–11.3 |
| | Confluent | 22 (4.0)§ | 9/25 (36) | 3.4¶ | 2.6–4.6 |
| **B. Measurements made at 30°C** | | | | | |
| HSF | Sparse | 60 (6.1) | 1/12 (8) | 8.1 | |
| | Confluent | 29 (7.5)§ | 5/12 (42) | 8.8 | |

Cells grown in sparse (1–3 $\times$ 10$^3$ cells/cm$^2$) or dense (3–5 $\times$ 10$^5$ cells/cm$^2$) conditions were removed from the culture dishes and labeled with tetramethyl-rhodamine conjugates of Fab fragments of monoclonal antibodies to class I MHC antibodies. Cells from either sparse or dense cultures showed uniform membrane staining. The lateral diffusion of the MHC antigens on suspended cells was measured as described in Materials and Methods. HSF, neonatal human skin fibroblasts; WI-38, diploid lung fibroblast cell strain; VA-2, SV40 transformed human fibroblast cell line; cl 1d, transformed mouse fibroblast cell line.
* SEM.
‡ Number showing no recovery/total (percent of total).
§ $P < 0.05$, one-sided Mann-Whitney-Wilcoxon U test.
¶ $P < 0.05$, Student's $t$ test based on ln values of diffusion coefficients. $s^2_{\ln x}$ ranged from 0.37 to 0.68. Confidence intervals were calculated as follows:

$$\exp [\bar{x}_{(\ln x)} \pm t_n - 1, 0.05 \, s^2_{(\ln x)}/n ].$$

that showed no recovery on the timescale of the experiment ($D < 1 \times 10^{-11}$ cm$^2$/s) and to a decrease in the percent recovery on cells that did show measurable recovery. This is shown more clearly in Fig. 1, which presents the distributions of recovery and diffusion coefficients measured on HSF cells. Cells that showed no recovery were not included in the histograms of diffusion coefficients. The mobile antigens on HSF cells from dense cultures showed similar diffusion coefficients to those for antigens on cells from sparse cultures. In contrast, diffusion coefficients of mobile antigens in cells from confluent cultures of the transformed cell lines, VA-2 and cl 1d, or from late passage WI-38 were significantly lower than the diffusion coefficients of antigens in cells from sparse cultures.

The fraction of cells with immobile MHC antigens was not changed when measurements were made at 30°C instead of 19°C (Table I [B]). No recovery was detected in 42% of dense HSF cells, while no recovery was measured in only 8% of sparse cells and the same diffusion coefficient was measured in both cell populations. Diffusion coefficients and percent recoveries of the lipid probe 3,3'-dihexadecylindo-carbocyanine iodide were not different in sparse and dense populations.

### Influence of Culture Age, Cell Density, and Culture Medium on Lateral Diffusion of MHC Antigens

Dense cultures differed from sparse cultures in their age, the extent of cell–cell contact, and the rate of cell proliferation,

**Table II. Effects of Cell Culture Conditions on the Lateral Diffusion of MHC Antigens: Effects of Cell Culture Age—cl 1D Cells**

| Culture conditions | Recovery | | Diffusion coefficient of mobile antigens ($\times 10^{10}$ cm$^2$/s) |
| | Average % recovery | Cells with no recovery | |
|---|---|---|---|
| Sparse-1 day | 32 (4.4) | 0/10 (0%) | 11.0 |
| Sparse-7 days | 28 (6.0) | 3/12 (25%) | 12.0 |
| Dense-7 days | 15 (3.1)* | 10/22 (45%) | 3.1‡ |

cl 1d cells were plated at 1 $\times$ 10$^3$ cells/cm$^2$ for 24 h or at 1 $\times$ 10$^2$ cells/cm$^2$ or 5 $\times$ 10$^4$ cells/cm$^2$ for 7 d. The lateral diffusion of the MHC antigens was measured on cells in suspension as described in Materials and Methods.
* $P < 0.05$, one-sided Mann-Whitney-Wilcoxon U test.
‡ $P < 0.05$, Student's $t$ test.

**Table III. Effects of Cell Culture Conditions on the Lateral Diffusion of MHC Antigens: Effects of Cell Contacts—VA-2 Cells on Coverslips**

| Contacts | % Recovery | Diffusion coefficient ($\times 10^{10}$ cm$^2$/s) |
|---|---|---|
| None | 62 (5.6) | 5.0 |
| Many | 43 (6.2)* | 4.1 |

VA-2 cells were plated at 5 $\times$ 10$^3$ cells/cm$^2$ on glass coverslips. After 48 h in culture, the lateral diffusion of the MHC antigens was measured on cells attached to coverslips.
* $P < 0.05$, one-sided Mann-Whitney-Wilcoxon U test.

**Table IV. Comparison of Measurements of VA-2 Cells in situ on Coverslips and Suspended from Coverslips**

| Measurement condition | Recovery | | Diffusion coefficient of mobile antigens ($\times 10^{10}$ cm$^2$/s) |
| | Cells with no recovery | Average % recovery | |
|---|---|---|---|
| Coverslips | 2/14 (14) | 56 (5.3) | 3.2 |
| Suspension | 4/13 (31) | 23 (4.7) | 5.0 |

VA-2 cells were plated on glass coverslips. After 4 d in culture, the lateral diffusion of the MHC antigens was measured as described in Materials and Methods on cells in situ on coverslips or after suspending the cells from the coverslips.

all of which can influence the lateral diffusion of membrane molecules (10, 13, 59). We examined the effects of each of these factors on the lateral diffusion of MHC antigens. Table II shows the measured diffusion coefficients and recoveries on cl 1d cells plated at very low density (1 $\times$ 10$^2$ cells/cm$^2$) or at high density (5 $\times$ 10$^4$ cells/cm$^2$) and maintained for 7 d with cells plated at low density for 24 h. Cells were stained and examined in suspension. Older cultures showed an increased proportion of cells exhibiting no measurable recovery. However, only cells maintained under dense conditions for 7 d exhibited significantly decreased diffusion coefficients and mobile fractions. The observed differences between sparse and dense cells were not due solely to reduced cell proliferation rates in dense cultures as the lateral mobility of the MHC antigens was not reduced on serum-arrested cells or cells grown for 24 h in media conditioned for 7 d by confluent cultures.

The effects of cell contact on lateral diffusion of MHC were examined on cells cultured and stained on glass coverslips. Measurements made on single VA-2 cells and cells in clusters were compared. The percent recoveries were

significantly reduced on cells with multiple cell contacts (Table III) when compared with cells showing no or few contacts. However, all cells examined showed measurable recovery and the diffusion coefficients on single cells and on cells with multiple contacts were not significantly different. The changes in lateral diffusion with cell contact affected the whole cell as recoveries and diffusion coefficients measured in areas of cell–cell contact were no different from measurements made on the free side of the same cell. Further, no differences were observed in measurements made on the dorsal and ventral surfaces of the same cell.

In general, diffusion coefficients were lower and recoveries were higher on cells examined in situ on coverslips compared with cells examined after suspension. Table IV shows data from VA-2 cells cultured on coverslips for 4 d. The number of cells exhibiting no mobile antigens was higher in cells examined in suspension. These results are consistent with reported observations on wheat germ agglutinin receptors on BALB/c 3T3 fibroblasts (51), although the differences observed for MHC antigens were much lower.

## Conversion from the Sparse to the Dense Phenotype

Cells examined immediately after suspension from dense cultures showed low recoveries, but the measured recoveries increased within 24 h after replating the cells in sparse conditions. To further examine the conversion from the low lateral mobility of dense cells to the higher mobility of sparse cells, we measured the lateral diffusion of MHC antigens on HSF cells from dense cultures at various times after replating under sparse conditions. As shown in Table V, cells resuspended 2 h after replating continued to exhibit reduced recoveries and no recovery was measured on 47% of the cells examined. However, by 6 h after replating, the measured recoveries were increased to values typically observed in cells from sparse cultures and were similar to values measured on cells examined 24 h after replating. The increase in lateral mobility appears to require spreading as cells cultured for 24 h on bacteriological plates in which cell spreading did not occur continued to exhibit low recoveries.

HSF cells were also replated on coverslips and examined in situ. Cells spread more slowly on untreated coverslips than on tissue culture dishes and only began to extend lamellae ∼2 h after plating. At this time, the centers of the cells continued to show low recoveries (Table VI), but the spread-

### Table V. Conversion of Immobile Receptors: HSF Cells Examined in Suspension

| | | Recovery | | |
|---|---|---|---|---|
| Time after replating | Substrate | Average % recovery | Cells with no recovery | Diffusion Coefficient of Mobile Antigens ($\times$ 10$^{10}$ cm$^2$/s) |
| 2 h | Adherent | 29* | 7/15 (47%) | 2.8‡ |
| 6 h | Adherent | 52 | 0/5 (0%) | 4.2 |
| 24 h | Adherent | 43 | 2/23 (9%) | 6.0 |
| 24 h | Nonadherent | 21* | 8/15 (53%) | 6.7 |

HSF cells were suspended from confluent cultures and plated on tissue culture dishes (adherent substrate) or on bacteriological petri plates (nonadherent conditions) for the indicated times. The cells were then resuspended and the lateral diffusion of the MHC antigens was measured.
*Values were significantly different from 24-h adherent condition based on Mann-Whitney-Wilcoxon U test.
‡P < 0.05, Student's t test comparing 2-h vs. 24-h adherent.

### Table VI. Conversion of Immobile Receptors: HSF Cells Examined on Coverslips

| | | Recovery | | |
|---|---|---|---|---|
| Time after replating | Location on cell | Average % recovery | Cells with no recovery | Diffusion Coefficient of mobile antigens ($\times$ 10$^{10}$ cm$^2$/s) |
| 2 h | center | — | 9/10 (90%) | — |
| | edge | 47 | 0/10 (0%) | 3.2 |
| 24 h | trailing | 52 | 0/6 (0%) | 2.1 |
| | leading | 62 | 0/6 (0%) | 3.7 |

HSF cells were suspended from confluent cultures and plated on glass coverslips for the indicated times. The lateral diffusion of the MHC antigens was measured on cells in situ.

ing edges of the cell exhibited high recoveries and diffusion coefficients characteristic of sparse cells. Differences were also observed in the lateral diffusion of MHC antigens on the leading and trailing edge of motile fibroblasts (Table VI). Although the diffusion coefficients measured at the two different places on the cell were less than a factor of two different, the values measured on the leading edge of the cell were always higher and the average ratio of the diffusion coefficient on the leading to the trailing edge was 1.9. Similar findings were observed for another fibroblast surface antigen, GP180, although the differences between diffusion coefficients measured on the leading and trailing edges were more pronounced for this antigen (29). Measured recoveries were not consistently higher on the leading edges of fibroblasts.

## Control of Lateral Diffusion by Extracellular Material Produced by Dense Cells

Because the production of extracellular matrix components changes when fibroblasts reach confluence (20, 31, 37), we examined the ability of ECM materials produced by confluent cells to affect the mobility of MHC antigens. Cells were plated at low density onto ECM from confluent cells. In all

### Table VII. Effects of Extracellular Materials on Lateral Diffusion of MHC Antigens

| | | Recovery | | Diffusion coefficient of mobile antigens ($\times$ 10$^{10}$ cm$^2$/s) |
|---|---|---|---|---|
| Cell type | Substrate | Recovery % (SEM) | Cells with no recovery | |
| HSF | Coverslips | 56 | 0/30 (0) | 3.2 |
| | HSF ECM | 33* | 8/27 (30) | 5.4‡ |
| | VA-2 ECM | 52 | 2/16 (13) | 3.4 |
| | WI-38 ECM | 28* | 3/10 (30) | 3.4 |
| VA-2 | Coverslips | 48 | 2/29 (7) | 3.2 |
| | VA-2 ECM | 24* | 17/39 (44) | 2.5‡ |
| | HSF ECM | 37 | 2/19 (11) | 4.9‡ |
| | WI-38 ECM | 33 | 0/10 (0) | 3.9 |
| WI-38 | Coverslips | 64 | 0/9 (0) | 3.7 |
| | HSF ECM | 35* | 2/10 (20) | 8.5‡ |
| | VA-2 ECM | 40* | 2/13 (16) | 3.1 |

ECM from the indicated cell type was prepared as described in Materials and Methods. Cells were plated at 5 × 10³ cells/cm² on glass coverslips or the indicated ECM. After 24–72 h, the lateral diffusion of the MHC antigens was examined on cells in situ as described in Materials and Methods.
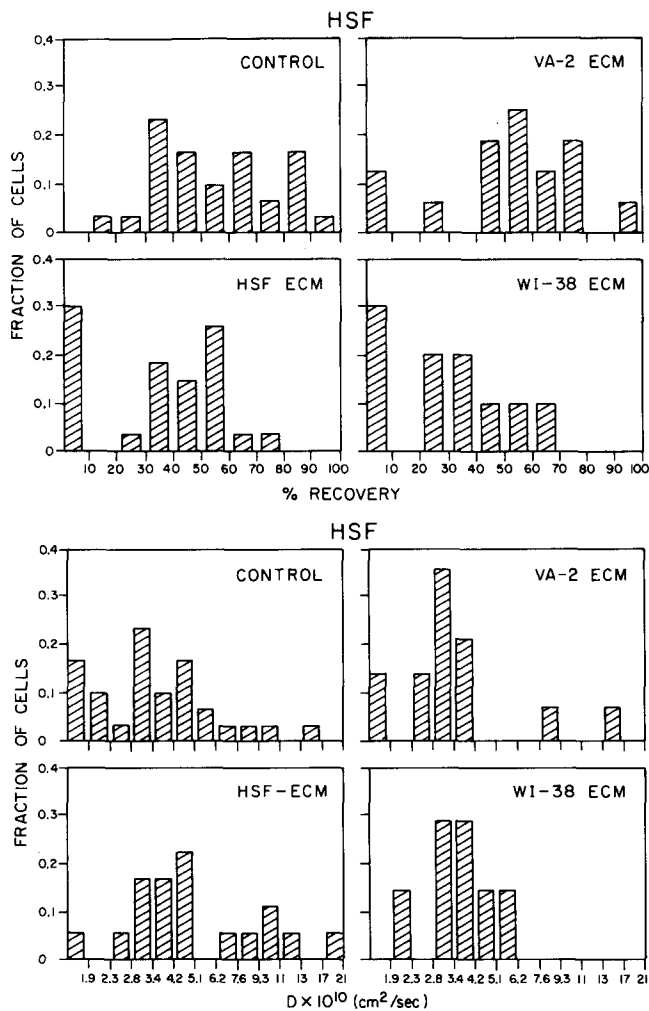*P < 0.05, Mann-Whitney-Wilcoxon U test.
‡P < 0.05, Student's t test based on 1 n values.

Figure 2. Effects of ECM on percent recoveries (top four panels) and diffusion coefficients (bottom four panels) of HSF cells.

Table VIII. Proteolytic Digestions of Extracellular Matrix

| Treatment | Recovery % (SEM) | Cells with no recovery | Diffusion coefficient of mobile antigens ($\times\ 10^{10}$ cm²/s) |
|---|---|---|---|
| None | 31 (4.7) | 11/41 (27) | 5.37 |
| Collagenase + trypsin + chicken serum | 40 (5.6)* | 1/17 (6) | 3.28‡ |
| Trypsin | 19 (4.4) | 14/28 (50) | 3.52 |
| Collagenase | 31 (6.7) | 4/14 (28) | 2.41‡ |
| Chicken serum | 27 (5.8) | 5/16 (31) | 5.40 |
| Trypsin + collagenase | – | 7/10 (70) | – |
| Trypsin + chicken serum | 25 (8.5) | 5/10 (50) | 2.97 |
| Collagenase + chicken serum | 50 (3.8)* | 0/10 (0) | 4.21 |
| Hyaluronidase | 49 (3.5)* | 0/12 (0) | 2.71‡ |
| Nitrous acid | 40 (4.2) | 0/8 (0) | 4.40 |
| Chondroitinase | 31 (10) | 2/7 (28) | 3.18 |

ECM was prepared from HSF cells then exposed to the indicated treatments as described in Materials and Methods. HSF cells were plated at low density (5 × 10³ cells/cm²) on the treated coverslips. After 24–72 h the lateral diffusion of the MHC antigens was measured on cells in situ as described in Materials and Methods.
* $P < 0.05$, one-sided Mann-Whitney-Wilcoxon U test.
‡ $P < 0.02$, Student's $t$ test on log values.

cases, measurements were made at the leading edges of well-spread fibroblasts. Shown in Table VII and Fig. 2 A, the average recovery was decreased on both HSF and VA-2 fibroblasts plated on ECM derived from confluent cultures of the same cell type, but was not decreased when cells are plated on ECM derived from the opposite cell type. This may be related to the transformed phenotype of VA-2 as ECM produced by WI-38, a second diploid cell line, induced a decrease in the recovery of MHC antigens on HSF cells but little change in recoveries on VA-2 cells. The background fluorescence was not increased in ECM-covered plates, so it is unlikely that residual antigen remaining associated with the ECM can account for the increased immobile fraction.

The effects of ECM on the diffusion coefficients of mobile MHC antigen molecules were less consistent. ECM from confluent VA-2 cultures induced a decrease in the diffusion coefficients of MHC antigens on VA-2 but not HSF or WI-38 cells. In contrast, all three types of cells showed higher diffusion coefficients when plated on ECM derived from HSF cells (Table VII).

To examine the nature of the material that was responsible for restriction of lateral diffusion, ECM-coated plates were treated with a series of enzymes as shown in Table VIII. Collagenase in the presence of chicken serum, but not col-

lagenase alone, abolished the effects of the ECM on the lateral mobility of the MHC antigens. Trypsin alone or in combination with only collagenase or chicken serum did not abolish and actually enhanced the effects of ECM on lateral mobility. Hyaluronidase also abolished the activity of the ECM, resulting in higher recovery and lower diffusion coefficients characteristic of cells plated on untreated coverslips. Mobility of MHC antigens in cells plated on ECM treated with chondroitinase ABC or with nitrous acid (which specifically degrades heparans) was not significantly different than that in cells plated on control ECM.

Purified ECM components did not induce restriction of the lateral diffusion of MHC antigens on fibroblasts. Cells plated on coverslips coated with collagens type I and III, laminin, or fibronectin and cells grown in the presence of fibronectin, laminin, heparin, or chondroitin sulfates showed no differences in recovery or diffusion coefficients compared to control cells. The diffusion coefficient of MHC antigens was increased in cells cultured in the presence of hyaluronic acid (control: $D = 3.05 \times 10^{-10}$ cm²/s; hyaluronic acid treated: $D = 5.12 \times 10^{-10}$ cm²/s), but the recoveries were not changed. This result is consistent with the observation that hyaluronidase treatment of HSF–ECM reduced the diffusion coefficients of MHC antigens to control values.

### Triton X-100 Residues

When cells cultured under sparse and dense conditions were incubated with ^{125}I-labeled KE-2 and then extracted with a Triton X-100–containing buffer under conditions that preserve the structure of the cytoskeleton (23), ~40% of the antibody remained associated with the residual cytoskeleton (Table IX). When cells were extracted with Triton X-100 in the presence of Buffer A which is known to disrupt cytoskeletal interactions (58), only 10% of the antibody remained associated with the residual fraction. No difference in the amount of insoluble antibody was observed between sparse

*Table IX. Antibody Remaining Bound after Triton X-100 Extraction*

| | WI-38 | | VA-2 | |
|---|---|---|---|---|
| Treatment | Sparse | Dense | Sparse | Dense |
| None | 36 ± 2 (4) | 40 ± 7 (4) | 39 ± 11 (3) | 37 ± 10 (3) |
| Buffer A | 23 ± 12 (4) | 12 ± 8 (3) | 15 (2) | 14 (2) |
| NaAzide/ | | | | |
| NaCN | 37 (1) | 40 (1) | 29 (1) | 31 (1) |
| A23187 | 52 (1) | 38 (2) | 32 (2) | 36 (2) |
| Concana- | | | | |
| valin A | 56 (1) | 69 (1) | 49 (2) | 51 (2) |

Cells were plated at high density and maintained for 7 d or at low density for 24 h. Cultures were labeled with $^{125}$I-KE2 in the presence of the indicated drugs for 2 h at 4°C and the amount of antibody remaining bound after treatment with Triton X-100–containing buffers was determined as described in Materials and Methods.

Values shown are expressed as the percent of antibody remaining bound after Triton X-100 treatment ± SD. The number of experiments in each case is indicated in parentheses. Values were corrected for nonspecific binding of antibody as measured in 100-fold excess of unlabeled antibody.

and dense cells of either the WI-38 or VA-2 cell lines. In addition, cells treated with A12387 or metabolic inhibitors, agents that affect lateral diffusion of MHC antigens (16), showed no change in Triton X-100–insoluble antibody binding. Treatment with concanavalin A at concentrations that induced complete immobility of MHC antigens increased the percent of bound antibody associated with the Triton X-100 residual fraction in both sparse and dense cells.

## Discussion

In this paper we have shown that the lateral diffusion of MHC antigens on the surface of cultured fibroblasts is modified by cell density. We report that increased cell density induces decreased lateral mobility reflected primarily as a decrease in the percent recovery. In three cell lines, the diffusion coefficient of the mobile molecules was also decreased in dense cultures. These changes in lateral diffusion in dense culture were associated with both increased cell age and cell–cell contact. In a previous study, there was no effect of cell density on the lateral mobility of a group of membrane proteins detected by a polyspecific antibody (22), although a high proportion of cells from tissue slices showed no mobile antigens. The difference in our results may reflect specific modulation related to the MHC antigens or may be due to differences between the cell types examined.

We have also presented data suggesting that the ECM may have a role in the restriction of lateral diffusion of MHC antigens. The major effect of ECM on lateral mobility of MHC antigens was a reduction in the fractional recovery of MHC antigens and an increase in the number of cells that showed no recovery on the time scale of the experiment. ECM materials produced by confluent cultures are generally both quantitatively and qualitatively different from the materials produced by sparse cells (20, 31, 37). Our experiments with digestive enzymes suggest that proteoglycans, particularly hyaluronic acid and an intact collagen matrix, are important in inducing the effects of ECM. ECM components have been implicated in control of cell polarity and regional restriction of cell surface components in other systems including acetylcholine receptors at the neuromuscular junction and mem-

brane receptors on epithelial cells (4, 19, 36, 43, 44). Complex ECM components also restricted the lateral mobility of specific membrane lipid probes (39, 41). Mild treatment of muscle cells with enzymes that degrade proteoglycans increases the mobile fraction but not the diffusion coefficient of soybean receptors (38). Together these results suggest that ECM can modulate the distribution of cell surface molecules by an effect on their lateral diffusion.

The data presented in this paper are consistent with a massive literature that has suggested the diffusion of membrane proteins is controlled by constraints other than overall lipid bilayer fluidity (1, 2, 15, 30, 38). The molecular basis for these constraints is not well understood and analysis is complicated by the fact that interpretation of fluorescence photobleaching data, particularly the percent recovery, is not completely straightforward. Several general treatments have appeared elsewhere (18, 29, 34, 35). Our data provide further evidence that at least two types of mechanisms with different lifetimes restrain lateral diffusion. One type of constraint is of long duration on the timescale of the fluorescence photobleaching and recovery experiment and is reflected in the mobile fraction. Other mechanisms of shorter lifetime restrain the diffusion of the mobile molecules. The specific molecular interactions involved in either type of restriction are not well understood and may vary from cell to cell. Cell–cell contact and cell–ECM interactions appear to affect primarily long duration interactions.

The effects of cell–cell contact and ECM materials on the lateral diffusion of MHC molecules could result from direct interactions between MHC antigens and the ECM either through specific attachments between MHC antigens and ECM components or through steric obstruction of the diffusion of MHC antigens by the ECM. While specific interactions between Class II MHC antigens and chondroitin sulfate proteoglycans have been reported (47) and integral membrane proteins that bind specific ECM components have been identified (12), purified components of the ECM did not have the same effect on fractional recovery as seen with complex cell-derived ECM. This suggests that multiple components or a highly cell type–specific component is required to modulate lateral diffusion. Modifications of the extracellular domains of membrane components such as glycosylation have generally resulted in changes in diffusion coefficients rather than mobile fractions (40, 56).

Our observations that changes induced by ECM are global and not specifically associated with distinct regions on the cell surface and that the conversion from the immobile to the mobile form requires spreading and occurs over time in culture suggest that major cellular rearrangements are involved. Thus, it seems likely that the ECM is acting by an indirect effect on the organization of cytoskeletal or membrane proteins. The ability of the ECM to induce changes in cytoskeletal arrangements is well documented in many cell types, and both specific and nonspecific changes in organization of cytoskeletal elements and the binding of cytoskeletal elements to membrane components have been reported (6, 8, 27, 32). Recent studies have shown that changes in the synthesis and distributions of intermediate filaments are induced by cell density and cell–cell interaction and have suggested that these changes are induced by interaction with ECM components (5). These changes closely parallel the types of

changes we have observed in lateral mobility of MHC antigens. ECM apparently restricts the lateral diffusion of a membrane lipid probe through changes in cell shape (41).

In some cells, MHC antigens are associated with an actin-containing matrix (33) and lateral diffusion on sparse cells is well below that of membrane proteins on cells treated to reduce cytoskeletal constraints. However, we observed that the fraction of Triton X-100–insoluble MHC antigens does not change in dense cells. These results are in contrast with studies on acetylcholine receptors and surface immunoglobulin in which decreased lateral diffusion was correlated with increased associations with Triton X-100–insoluble residues (50, 55). Evidence from a number of studies have suggested that the interactions of MHC antigens and the cytoskeleton may be different from that of other proteins. The MHC antigens on lymphocytes are more resistant to capping than several other lymphocyte surface antigens (7) and are not induced to associate with the Triton X-100–resistant cytoskeleton when cross-linked with antibodies (55). Localized binding of concanavalin A on lymphocytes globally reduces the lateral mobility of sIG but does not affect the MHC antigens (26). It has recently been reported that MHC antigens with truncated tails do not show increased lateral diffusion (17). These observations limit the types of associations that can restrict the lateral mobility of the MHC antigens.

Increased cell density is associated with other changes in cell surface dynamics. Patching, capping, and internalization of some surface receptors are decreased in confluent cultures (53) and the response to many hormones and growth factors is changed. In this paper we have shown that increased cell density possibly mediated through ECM components can influence the lateral diffusion of MHC molecules. Further examination of the basis for these changes may provide important insight into the dynamic regulation of membrane proteins.

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