THE RAPID INTERMIXING OF CELL SURFACE ANTIGENS AFTER FORMATION OF MOUSE-HUMAN HETEROKARYONS

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SUMMARY

Cells from established tissue culture lines of mouse (c11D) and human (V/A-2) origin were fused together with Sendai virus, producing heterokaryons bearing both mouse and human surface antigens which were then followed by the indirect fluorescent antibody method. Within 40 min following fusion, total mixing of both parental antigens occurred in over 90% of the heterokaryons.

Mouse H-2 (histocompatibility) and human surface antigens were visualized by successive treatment of the heterokaryons with a mixture of mouse alloantiserum and rabbit anti-V/A-2 antiserum, followed by a mixture of fluorescein-labelled goat anti-mouse IgG and tetramethylrhodamine-labelled goat anti-rabbit IgG(Fc).

The c11D × V/A-2 fusions were carried out in suspension and maintained at 37°C in a shaking water bath; aliquots were removed at various intervals and stained with the above reagents. The heterokaryon population was observed to change from an initial one (5-min post-fusion) of non-mosaics (unmixed cell surfaces of red and green fluorescence) to one of over 90% mosaics (total intermixing of the 2 fluorochromes) by 40 min after fusion. Mouse–human hybrid lines, derived from similar fusions, gave fluorescence patterns identical to those of the mosaic heterokaryons.

Four possible mechanisms would yield such results: (i) a very rapid metabolic turnover of the antigens; (ii) integration of units into the membrane from a cytoplasmic precursor pool; (iii) movement, or ‘diffusion’ of antigen in the plane of the membrane; or (iv) movement of existing antigen from one membrane site into the cytoplasm and its emergence at a new position on the membrane.

In an effort to distinguish among these possibilities, the following inhibitor treatments were carried out: (1) both short- and long-term (6-h pre-treatment) inhibition of protein synthesis by puromycin, cycloheximide, and chloramphenicol; (2) short-term inhibition of ATP formation by dinitrophenol (DNP) and NaF; (3) short- and long-term inhibition of glutamine-dependent pathways with the glutamine analogue 6-diazo-5-oxoornithine; and (4) general metabolic suppression by lowered temperature.

The only treatment found effective in preventing the mosaicism was lowered temperature, from which resulted a sigmoidal curve for per cent mosaics versus incubation temperature. These results would be consistent with mechanisms iii and/or iv but appear to rule out i and ii.

From the speed with which the antigen markers can be seen to propagate across the cell membrane, and from the fact that the treatment of parent cells with a variety of metabolic inhibitors does not inhibit antigen spreading, it appears that the cell surface of heterokaryons is not a rigid structure, but is ‘fluid’ enough to allow free ‘diffusion’ of surface antigens resulting in their intermingling within minutes after the initiation of fusion.

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INTRODUCTION

The surface membranes of animal cells rapidly change shape as the cells move, form pseudopods, or ingest material from their environment. These rapid changes in shape suggest that the plasma membrane itself is fluid, rather than rigid in character, and that at least some of its component macromolecules are free to move relative to one another within the fluid. We have attempted to demonstrate such freedom of movement using specific antigen markers of 2 unlike cell surfaces. Our experiments show that marker antigens on surface membranes spread rapidly when unlike cells are fused. The speed of antigen spread and its insensitivity to a number of metabolic inhibitors offer some support for the notion of a fluid membrane.

We have approached the problem of mixing unlike, and hence readily differentiated, cell surface membranes by using Sendai virus to fuse tissue culture cells of mouse and human origin (Harris & Watkins, 1965). The antigens of the parent cell lines and of progeny heterokaryons have been visualized by indirect immunofluorescence, using heteroantisera to whole human cells, and alloantiserum to mouse histocompatibility antigens. Both sera were cytotoxic for intact cells in the presence of complement; alloantisera have previously been shown, by both immunofluorescence and immunoferritin techniques, to bind only to the surface of intact cells (Möller, 1961; Cerottini & Brunner, 1967; Drysdale, Merchant, Shreffler & Parker, 1967; Davis & Silverman, 1968; Hammerling et al. 1968).

The surface antigens of heterokaryons between hen erythrocytes and HeLa cells and between Ehrlich ascites and HeLa cells have previously been studied using mixed agglutination techniques for antigen localization (Watkins & Grace, 1967; Harris, Sidebottom, Grace & Bramwell, 1969). In these studies intermixing of surface antigens was demonstrable within an hour or two of heterokaryon formation. However, the antigens could not readily be localized, since the marker particles used were several microns in diameter; also, observations were not made of the earliest time at which mixing occurred. We have been able to examine heterokaryons within 5 min of their formation and to show that antigen spread and intermixing occurs within minutes after membrane fusion. Studies on cells poisoned with a variety of metabolic inhibitors strongly suggest that antigen spread and intermixing requires neither de novo protein synthesis nor insertion of previously synthesized subunits into surface membranes.

MATERIALS AND METHODS

Cell lines

ciD. A thymidine-kinase negative (TK-) subline of the mouse 'L' cell, isolated by Dubbs & Kitt (1964), and kindly provided by Dr H. G. Coon.
VA-2. An 8-azaguanine-resistant subclone, isolated by Weiss, Ephrussi & Scaletta (1968), obtained from W-18-VA-2, an SV40-transformed human line which has been free of infective virus for several years (Ponten, Jensen & Koprowski, 1963).
SaI. An ascites tumour (designated as Sarcoma I), provided by Dr A. A. Kandutsch, The Jackson Laboratory, and carried in A/J mice; it was used as a convenient source of mouse cells for absorption of antiglobulin reagents.
Tissue culture

The c11D and VA-2 lines were routinely grown in a modified F-12 medium containing 5% foetal calf serum (FCS) (Coon & Weiss, 1969) or in Minimal Essential Medium with 5% FCS, 5% Fungizone and 100 units penicillin/ml. The cultures were maintained at 37°C in a water-jacketed CO2 incubator, 98% humidity, 5% CO2.

For experiments or routine passages, cells were harvested with 2.5% heat-inactivated chicken serum, 0.2% trypsin and 0.002% purified collagenase (Worthington CSL) in Moscona's (1961) solution, which is referred to as 'CTC'.

Sensitizing antibodies

Mouse alloantiserum (FAS-2). Preparation: antibodies primarily directed against the H-2k histocompatibility antigens were obtained by a series of intraperitoneal injections of CBA/J (H-2k) mouse mesenteric lymph node and spleen cells into BALB/cJ (H-2d) mice (4-recipients: 1 donor). Six injections were given twice weekly, followed by a booster 2 weeks after the last injection. The animals were bled from the retro-orbital sinus 4 and 5 days post-booster.

Specificity. Reaction with mouse cells (c11D): Aliquots of 2.5 x 10^6 c11D cells were treated in suspension with 0.1 ml of two-fold dilutions of FAS-2 from 1/10 to 1/80. The cells were agitated periodically for 15 min at room temperature at which time they were washed twice in phosphate-buffered saline (PBS). They were then resuspended in 0.05 ml of fluorescein-labelled rabbit antimouse IgG, incubated, and washed as above. The cells were then put on to Vaseline-ringed slides, covered and observed in the fluorescence microscope. Ring reactions as reported by Moller (1961) were observed with decreasing brightness upon increasing dilutions of the FAS-2. As maximum brightness was desired, the 1/10 dilution was chosen for all subsequent staining reactions.

Reaction with human cells (VA-2): No fluorescence was observed when analogous staining reactions were carried out with human cells.

Reaction with Sendai virus: It was discovered that VA-2 cells pre-treated with Sendai virus became positive for the FAS-2 sensitization. Normal mouse sera from BALB/cJ, CBA/JJ, DBA/2J and A/JJ strains were also shown to exhibit this anti-Sendai activity. This activity in FAS-2 was easily absorbed by treating a 1/5 dilution of the antiserum with 333-666 haemagglutinating units (HAU)/ml of virus for 30 min at room temperature and overnight at 4°C. The absorbing virus was then removed by centrifugation.

Rabbit anti-VA-2 antiserum (RaVA-2) preparation: VA-2 cells were grown in Falcon plastic Petri dishes, harvested with CTC, and washed 3 times in Hank's balanced salts solution, BSS (HEPES-buffered) to remove the foetal calf serum. 2 x 10^6 cells were emulsified with Freund's complete adjuvant (cells : adjuvant = 1:2) and injected intradermally (flanks and footpads) into a New Zealand white rabbit. One week later 10^6 washed cells were given intradermally (flanks only; 10^6 cells/site). The rabbit was bled from the ear vein 1 and 2 weeks following this second injection. The sera were heat-inactivated at 56°C for 30 min, aliquoted and stored at -30°C.

Specificity of RaVA-2: Reaction with VA-2: VA-2 cells were seeded on to coverslips (2.5 x 10^3/coverslip) and allowed to adhere and spread. The coverslips were then washed with Hank's and 0.1 ml of 2-fold dilutions of the RaVA-2 from 1/2 to 1/256 were added. After incubation in a moist chamber for 15 min at room temperature, the coverslips were washed twice in Hanks's BSS and similarly treated with tetramethylrhodamine (TMR)-labelled goat anti-rabbit IgG (anti-Fc). The cells gave strong fluorescent ring reactions at the lower dilutions of the sensitizing antibody; the 1/4 dilution was chosen for all subsequent staining reactions.

Reaction with c11D: When analogous staining reactions were carried out with the mouse cells, a very weak fluorescence was seen in the lower dilutions of the RaVA-2. Consequently, the serum was routinely absorbed with 5 x 10^6 c11D/ml of a 1/2 diluted serum (30 min at room temperature).

Reaction with Sendai virus: The c11D cells, when pre-treated with Sendai virus, gave weak positive staining with the mouse-absorbed RaVA-2. Therefore, the RaVA-2 was also absorbed with 333 HAU/ml (30 min at room temperature and overnight at 4°C). This doubly absorbed RaVA-2 then gave a negligible background on the c11D cells.
Fluorescent antibodies

Goat anti-mouse IgG. Preparation of mouse IgG: A 16% Na₂SO₄ cut of 26 ml of normal BALB/c serum was dissolved in 8 ml of 0.2 M NaCl, 0.1 M phosphate buffer, pH 8.0 and then dialysed against this buffer in the cold prior to chromatography on a 2.5 x 100 cm column of Sephadex G-200, in the same buffer. Included fractions comprising the second protein peak off the column were pooled and dialysed against 0.01 M phosphate buffer, pH 7.5. The dialysed material was applied to a 500-ml column of DEAE-cellulose, equilibrated with 0.01 M phosphate buffer. Material eluting stepwise from the column in 0.025 and 0.05 M phosphate buffer was pooled and concentrated in an Amicon ultrafilter. The purified material showed only an IgG arc upon immunoelectrophoresis on agarose and reaction with rabbit anti-whole mouse serum.

Immunization of goat: 10 mg of immunogen was emulsified with Freund's complete adjuvant (immunogen: Freund's = 1:2) and injected intramuscularly into a 6-month-old goat. Three and one-half weeks later, 500 ml of blood were collected from the jugular vein and the serum tested by immunoelectrophoresis. Even though the immunogen had shown no contaminants as judged by the rabbit anti-whole mouse serum, a trace amount of a more negative protein was present. Though the goat anti-mouse IgG was not monospecific, non-specificity was not observed in the indirect fluorescent antibody technique described under sensitizing antibodies.

Conjugation to FITC: The isothiocyanate derivative of fluorescein (FITC) was used for conjugation to partially purified goat antibodies, employing the method of Wood, Thompson & Goldstein (1965). The fluorescein-labelled antibodies were eluted stepwise from DEAE-cellulose with 0.05, 0.1, 0.2 and 0.3 M phosphate buffers, pH 7.5. The 0.1 M phosphate buffer cut, having an O.D. 280/495 = 2.0 and a protein concentration of 1.3 mg/ml, was used in all our experiments.

Goat anti-rabbit IgG (Fc). Source: Goat anti-rabbit IgG (Fc), prepared against the Fc portion of the gamma heavy chain, was kindly provided by Dr J. J. Cebra.

TMRITC conjugation: The preparation of tetramethylrhodamine (TMR)-labelled antibodies was carried out under the same conditions as for the fluorescein conjugation. An 0.1 M phosphate buffer cut from DEAE-cellulose had an O.D. 280/515 = 1.7 and a protein concentration of 1 mg/ml; it was used in all studies described here.

Sendai virus

The Sendai virus used in the experiments described in this report, was kindly provided by Dr H. G. Coon. Its preparation was as published (Coon & Weiss, 1969) except that the virus was inactivated with β-propiolactone, rather than by ultraviolet irradiation.

Formation of heterokaryons

Heterokaryons were produced by the suspension fusion technique originally described by Okada (1962) for homokaryons. The parental ratios were c11D/VA-2 = 2:4; 3 x 10⁶ cells were resuspended in 0.1 ml of cold Sendai (100-250 HAU/ml) and shaken at 0-4°C for 10 min and then at 37°C for 5-10 min. Culture medium was then added for a 10-fold dilution of the cells.

Formation of hybrid cell lines

Mouse-human hybrid cell lines were produced by viral fusion as for the heterokaryons. Following fusion, the cells were plated at 3 x 10⁶/ml in normal medium; 24 h later this medium was replaced by ‘HAT’ (Littlefield, 1964), which was used for all subsequent feedings of these plates and the resulting hybrid lines.
Fluorescent staining of cells

Cells from fusion experiments or hybrid cells were washed in Hanks's BSS and resuspended in a mixture of sensitizing antibodies: FAS-2 and RaVA-2 (1/10 and 1/4 final dilutions, respectively), 0.1 ml mixture/3-6 x 10^6 cells; incubation at room temperature for 15 min. Finally, the cells were washed twice in Hanks's BSS, resuspended in a small volume of the same, placed on a Vaseline-ringed slide, and observed in the fluorescence microscope.

Table 1. Filter combinations used for excitation of fluorescein and tetramethylrhodamine conjugates

<table>
<thead>
<tr>
<th>Filter type</th>
<th>Fluorochrome</th>
<th>For observation of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Excitation</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>Blue interference filter</td>
<td>Type: PAL</td>
<td>No.: 100-105</td>
</tr>
<tr>
<td>λ max.: 437 nm</td>
<td>T max.: 43 %</td>
<td>λ max.: 545 nm</td>
</tr>
<tr>
<td>HW = 21 nm</td>
<td>Source: Schott and Gen., Mainz</td>
<td>HW = 21 nm</td>
</tr>
<tr>
<td>Barrier or Window</td>
<td>Kodak Wratten gelatin</td>
<td>Kodak Wratten gelatin</td>
</tr>
<tr>
<td></td>
<td>Filter no. 58 (green)</td>
<td>Filter no. 23 A (red) and</td>
</tr>
</tbody>
</table>
| or Kodak Wratten gelatin | RG-1 filter (Red; 2 x 17 mm) | (Schott and Gen., Mainz)
| Filter no. 8 K 2 (yellow) |

Fluorescence microscopy

All observations of fluorescent cells were made with a Leitz Ortholux Microscope, using darkfield condenser D 1-20 and an Osram HBO 200-W high-pressure mercury lamp as the light source. The exciting light was first passed through a Corning BG-38 heat filter and then through a combination of interference and barrier filters, depending upon the type of fluorescence to be maximized (see Table 1). The interference filters were patterned after those reported by Ploem (1967) to give maximum brightness for fluorescein- and tetramethylrhodamine-labelled antibodies.

Photography

Pictures of cells stained with fluorescent-labelled antibodies were taken using a Leica camera and exposing Anscochrome 200 daylight film (ASA 200) for 3-4 min or Hi-Speed Ektachrome (ASA 160) for 4-6 min.

RESULTS

Staining of c11D and VA-2

When c11D or VA-2 populations were stained for either H-2 or human antigens by the protocol given in Materials and Methods, 2 basic fluorescent patterns were observed: (1) The majority of the cells gave a full ring reaction, (Möller, 1961) with
intensities varying from cell to cell; an occasional cell gave no fluorescence except for a weak blue-green autofluorescence which was easily distinguishable from the FL or TMR fluorescence. On the cells giving ring reactions, distinct, tiny patches of fluorescence could be seen by focusing on the upper or lower cell surfaces. When a cell was in focus for the ring reaction (that is, at the cell equator), these patches were no longer visible. (2) Some cells gave only a partial ring reaction, and upon focusing on the upper or lower surfaces, the patches of fluorescence were in highest concentration on the cell half giving the partial ring reaction.

Staining of \( c11D \times VA-2 \) hybrid cell lines

Hybrids between \( c11D \) and \( VA-2 \) were produced as described in Materials and Methods. Colonies of hybrid cells first appeared 13 days following fusion. Six of these were isolated and stained to provide positive controls for doubly antigenic cells (Fig. 3 A, B). Cells of all lines fluoresced green, indicating a content of mouse H-2 antigens, and the intensities were comparable to those given by the mouse parent, \( c11D \). The intensity of the red fluorescence, marking human antigens, varied from line to line. If the human parent, \( VA-2 \), is given an arbitrary intensity of + + units, then the hybrid lines gave the following: \( MH-1 \), + +; \( MH-5 \), +; \( MH-2 \), ±; and \( MH-6 \), −.

Time-course of staining of heterokaryons

Fusions and fluorescent antibody staining of \( c11D \times VA-2 \) were carried out as described in Materials and Methods. Crosses of \( c11D \times c11D \) and \( VA-2 \times VA-2 \) were also made as controls for antibody specificity. Following the fusion reaction (5–10 min at 37 °C), the cells were diluted 10-fold with medium and shaken at 37 °C. Aliquots removed at various times were stained immediately, and the stained cells were kept at 0 °C until observation.

Though cell fusion indices were not measured, the degree of fusion in the system was not great, as evidenced by the low number of double-staining cells obtained; the scanning of several fields was required to count adequate numbers of the heterokaryons.

The control slides of \( c11D \times c11D \) and \( VA-2 \times VA-2 \) showed cells with ring reactions of only one colour; green for \( c11D \) and red for \( VA-2 \). No double-staining cells were seen.

Four basic types of double-staining cells were observed in the \( c11D \times VA-2 \) crosses: (1) \( M\frac{1}{2}H\frac{1}{2} \), a heterokaryon showing unmixed partial ring reactions for each fluorochrome; (2) \( M\frac{1}{2}H1 \), in which the heterokaryon showed a complete ring reaction for the human antigens, but only a partial one for the mouse H–2 antigenic markers; (3) \( M1H\frac{1}{2} \), the reverse of the pattern seen in \( M\frac{1}{2}H1 \); this type was much rarer than \( M\frac{1}{2}H1 \), and (4) \( M1H1 \), which we term mosaics, showing complete ring reactions for both fluorochromes (Fig. 3 c–g). In addition to these 4 types, a large number of parent cells were seen which had not fused; these provided another control for reagent specificity. Homokaryons, though undoubtedly present, would not be detected since only one type of fluorescence would be seen on such cells. Also present were weakly
staining cells which could not be categorized, as well as damaged cells; the latter were characterized by a diffuse fluorescence of both fluorochromes throughout the cell.

Table 2 shows the results obtained up to 2 h following the initial fusion reaction. There is a definite trend from an initial population of non-mosaics to one of over 90% mosaics (as percentage of double-stained cell population) by 40 min. Fig. 1 shows a bar graph of the population shift over time.

### Table 2. Time course of antigen spread

<table>
<thead>
<tr>
<th>Incubation time at 37 °C (min)</th>
<th>Double-staining category</th>
<th>Total</th>
<th>Mosaics %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mi-Mi</td>
<td>Mi-Hi</td>
<td>M1-Hi</td>
<td>M1-H1</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>4</td>
<td>0</td>
</tr>
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<td>20</td>
<td>0</td>
</tr>
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<td>0</td>
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</tr>
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<td>0</td>
<td>2</td>
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</tr>
<tr>
<td>40</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 1. Appearance of completely double-staining (mosaic) cells in the population of fused cells.
In initial studies in which the fused cells were placed on coverslips in Petri dishes and allowed to adhere and spread for up to 12 h before staining, all double-staining cells observed were mosaic in appearance.

Inhibitor studies

The rapid spread of antigens across the surfaces of heterokaryons could be due either to movement of antigens across the cell surface ('diffusion') or to new antigen synthesis. In an attempt to distinguish between these possibilities, cells were treated with various metabolic inhibitors before and during fusion. Since the time-course studies showed that over 90% of the double-stained cells were mosaic by 40 min at 37 °C, this time period was chosen for testing the effects of the inhibitors. The experiments fall into 4 categories: inhibition of protein synthesis, of ATP formation, of glutamine-dependent synthetic pathways, and generalized metabolic inhibition by lowered temperature.

Table 3. Effect of inhibition of protein synthesis on mosaicism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Double-staining category</th>
<th>M_{1/2}H_{1/2}</th>
<th>M_{1/2}H_{1}</th>
<th>M_{1}H_{1/2}</th>
<th>M_{1}H_{1}</th>
<th>Total</th>
<th>Mosaics %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puromycin, short term</td>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>35</td>
<td>36</td>
<td>97</td>
</tr>
<tr>
<td>Puromycin, long term</td>
<td></td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>24</td>
<td>33</td>
<td>73</td>
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<tr>
<td>Cycloheximide, short term</td>
<td></td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>43</td>
<td>45</td>
<td>95</td>
</tr>
<tr>
<td>Cycloheximide, long term</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>Chloramphenicol, short term</td>
<td></td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>34</td>
<td>41</td>
<td>83</td>
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<tr>
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<td>2</td>
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<td>39</td>
<td>41</td>
<td>95</td>
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<tr>
<td>Chloramphenicol, short term</td>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>40</td>
<td>41</td>
<td>98</td>
</tr>
<tr>
<td>Chloramphenicol, long term</td>
<td></td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>43</td>
<td>50</td>
<td>86</td>
</tr>
</tbody>
</table>

Inhibition of protein synthesis. Cycloheximide and puromycin were tested for potency in terms of the inhibition of [3H]leucine incorporation into TCA-precipitable material. Cycloheximide was found to give over 95% inhibition at 5 μg/ml after 30 min incubation, whereas comparable inhibition for our batch of puromycin required 80 μg/ml as measured after 3 h.

For the initial experiments the parent cells were suspended in medium containing either puromycin (80 μg/ml) or cycloheximide (5 μg/ml) and incubated for 15 min at 37 °C. The cells were then pelleted and fusion carried out as usual. After the fusion reaction, the cells were diluted 10-fold with inhibitor-containing medium and shaken for 30 min at 37 °C before staining. Table 3 shows the results of these and other inhibitor experiments. Neither inhibitor had any effect on mosaic formation.

If membrane or antigen subunits were synthesized some time prior to being released to the surface, short-term inhibition of protein synthesis might be without effect on
such subunits, or their insertion into membrane. Therefore, the parent cells were treated with inhibitors for 6 h prior to their fusion. Confluent plates of ciliD and VA-2 were treated with inhibitor in the concentrations used in short-term experiments, but for 6 h; after 3 h incubation, the medium was replaced with fresh inhibitor-medium to ensure continued inhibitor potency. (Both inhibitors had been tested with [3H]-leucine for prolonged inhibition, and they did retain their potency during a 3-h incubation.) Two and one-half hours later, the cells were harvested with CTC-inhibitor, counted and checked for viability (nigrosin dye exclusion test). Viability was greater than 95%. The experiment was then continued, using the same procedures as for the short-term inhibition experiments. Table 3 gives the results of these experiments. In the initial long-term experiment, a slight effect for both inhibitors was noted; however, a repeat experiment gave results equal to control values.

Table 4. Effect of inhibition of glutamine-dependent pathways on mosaicism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Double-staining category</th>
<th></th>
<th></th>
<th></th>
<th>Total</th>
<th>Mosaics %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON, short term</td>
<td>M⁺⁻H⁻</td>
<td>M⁻⁻H⁻</td>
<td>M⁻⁻H⁺</td>
<td>M⁺⁻H⁺</td>
<td>Total</td>
<td>Mosaics %</td>
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<tr>
<td>DON, long term</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>44</td>
<td>45</td>
<td>98</td>
</tr>
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</table>

Analogous short and long-term inhibition experiments were also carried out with chloramphenicol, at a concentration of 200 µg/ml (Table 3). Again negligible inhibition of mosaic formation was noted.

Inhibition of ATP formation. When cells are exposed to 2.5 x 10⁻³ M DNP + 2 x 10⁻³ M NaF following initiation of fusion for 10 min at 37 °C, their content of ATP, as measured in terms of the light flash produced in a luciferin/luciferase system, (Denburg, Lee & McElroy, 1969) falls to 20% of that of control cells 5 min after addition of the uncouplers and to 17% at 10 min. After shaking for a further 30 min at 37 °C, the cells were stained and observed. DNP+NaF did not inhibit mosaic formation.

Inhibition of glutamine-dependent synthetic pathways. Fusion experiments were performed using cells treated with the glutamine analogue, DON (6-diazo-5-oxonorleucine), at a concentration of 250 µg/ml in the medium which contained 292 µg/ml of L-glutamine. In the short-term inhibition experiment, the parent cells were incubated in the presence of DON for 30 min at 37 °C prior to the fusion step. Following a fusion step of 5 min, the cells were diluted 10-fold in DON-containing medium, shaken for 35 min at 37 °C, stained and observed. Table 4 gives the results of this experiment, in which no effect was seen.

A long-term inhibition experiment was also performed, differing from the former only in that the cells, in culture, had been treated with DON 6 h prior to the fusion reaction. After 3 h in culture, the DON-containing medium was replaced with fresh inhibitor-medium to insure continued inhibition. Before fusion the cells were tested for viability (nigrosin dye exclusion); viability was greater than 95%. The results of
L. D. Frye and M. Edidin

this experiment are given in Table 4. Again, mosaic development proceeded as normal.

Temperature studies

To see the effects of lowered temperature upon mosaic formation, aliquots of cells were maintained for 30 min at 0, 15, 20 and 26 °C after an initial 10-min fusion step. The results are tabulated in Table 5 and Fig. 2 shows a plot of temperature vs. per cent mosaicism in the double-stained cell population. Lowered temperature does appear to inhibit antigen spread and mixing.

In all the inhibition experiments described, the degree of cell fusion did not appear to be significantly reduced by any of the inhibitors.

Table 5. Effect of temperature on antigen spread and mosaicism

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Double-staining category</th>
<th>Total</th>
<th>Mosaics %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_{1}^{-}-H_{1}^{+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$M_{1}^{-}-H_{1}^{+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>0*</td>
<td>18</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>15*</td>
<td>22</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>7</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>20*</td>
<td>1</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>26*</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

*5-min fusion at 37 °C.

Fig. 2. Effect of temperature on the appearance of mosaic cells within 40 min of cell fusion.
DISCUSSION

Surface antigens of cuD and VA-2 cells and of their stable hybrids

Fluorescent antibody staining of cells of lines cuD and VA-2 showed surface antigens homogeneously distributed, in tiny patches, over the entire surface of most of the cells observed. The appearance of a 'ring reaction' (Möller, 1961), seems to be due to surface membrane curvature, resulting in a greater number of fluorochromes observed per unit area when focusing in the plane of a cell's equator than when focusing on its upper or lower hemispheres. The reason why some cells exhibit a partial ring reaction at the equator remains unclear; similar results were noted by Möller (1961).

The discrete patches of fluorescence seen away from the cell equator indicate that surface antigens are localized or clustered, rather than spread through the entire surface. Others have reported similar observations, using both light and electron microscopy. Cerottini & Brunner (1967), using an indirect fluorescent antibody method to detect mouse H-2 alloantigens on various tumour and normal cells, observed large patches of fluorescence, especially in ethanol-fixed cells. Two groups using ferritin-labelled antibodies have also shown H-2 antigens to be clustered in the membrane (Davis & Silverman, 1968; Hammerling et al. 1968).

Staining of hybrid lines between cuD and VA-2 provided a control for the appearance, when stained, of a cell whose surface is a mosaic of both human and mouse antigens, and is presumably synthesizing these antigens. Those lines which did stain positive for both sets of antigens were indistinguishable from the mosaic heterokaryons found 40 min after initiation of cell fusion.

Surface antigens of heterokaryons

The time-course study of antigens on the surface of newly formed heterokaryons showed that over 90% of double-staining cells are completely mosaic by 40 min after initiation of fusion. Though for technical reasons, a single cell cannot be followed through the various stages of mosaic formation, the population study would indicate a progression from $M_{\frac{1}{2}}-H_{\frac{1}{2}} \rightarrow M_{\frac{3}{4}}-H_{\frac{1}{2}} \rightarrow M_{1}-H_{1}$. The observation that the mouse and human antigens do not spread at the same rate thus giving rise to the $M_{\frac{3}{4}}-H_{1}$ class, remains unexplained. One possible reason for the apparently faster rate of human antigen mixing could be due to a concentration effect; that is, the human marker represents all VA-2 surface components antigenic for rabbits (for whole VA-2 cells were used in the rabbit immunization) whereas the mouse marker represents only H-2 alloantigens (which could presumably be in lesser amount per unit area than total human antigens per unit area on the VA-2 membrane). Those rare cells showing the reverse pattern, $M_{1}-H_{\frac{3}{4}}$, could possibly be explained on the basis of the fusion of one VA-2 cell with several cuD; cell nuclei were difficult to see under dark-field illumination for verification of multicellular fusion.

Four processes might account for the observed development of a mosaic pattern of antigen distribution: (i) a rapid synthesis of additional antigens, or a rapid metabolic turnover of existing antigens; (ii) integration of subunits, previously synthesized within the cell, into the membrane; (iii) movement or 'diffusion' of antigen within

Rapid intermixing of surface antigens
the plane of the membrane; or (iv) movement of existing antigen from one membrane site into the cytoplasm and its emergence at a new position on the membrane. Mechanisms iii and iv are difficult to distinguish operationally from each other, while it ought to be possible to distinguish i or ii from iii and iv.

If (i), rapid metabolic turnover, or synthesis of additional antigen molecules is responsible for the antigen redistribution observed, then one should be able to block the process with a suitable inhibitor. Unfortunately, lack of information on the chemistry of the antigens detected in our system precludes a definitive statement that antigen synthesis or replacement from a cytoplasmic precursor pool is inhibited by any of the inhibitors tested.

Short-term inhibition of protein synthesis, using puromycin, cycloheximide and chloramphenicol had no effect upon mosaicism. Three different inhibitors were used in an effort to block as many different sites of protein synthesis as possible. Unless one postulates that membrane proteins are synthesized in a metabolic compartment protected from all the inhibitors used, it would appear that rapid de novo protein synthesis is not a requirement for mosaic formation; indeed, other workers have shown that the inhibitors we used are effective in blocking the synthesis of membrane-associated molecules. Kraemer (1966, 1967) found puromycin effective in preventing the reappearance of sialic acid on Chinese hamster cells which had previously been treated with sialidase. Warren & Glick (1968) also found that puromycin blocked the turnover of \[^{14}C\]glucose in L cell membranes; cycloheximide retards incorporation of \[^{14}C\]leucine in chloroplast membrane protein of Chlamydomonas (Hoober, Siekevitz & Palade, 1969).

The glutamine analogue, DON was also without effect on antigen re-arrangement, both in short- and long-term inhibition experiments. DON has been shown to be an effective inhibitor of glutamine-utilizing amino transferase reactions involved in purine and pyrimidine synthesis and in amino sugar synthesis (summarized by Meister, 1962). In each of the systems, the inhibitor has been shown to be effective in the presence of a large excess of glutamine. We feel that in our experiments its failure to inhibit appears to rule out de novo synthesis of oligosaccharides and their attachment to pre-existing protein chains. The presence of amino sugars and sialic acid on the cell surface is well documented (see Cook, 1968) and indeed, DON has been found to inhibit another process involving cell surfaces, glutamine-dependent reaggregation of dissociated mouse embryoid body cells, when used in the presence of glutamine (Oppenheimer, Edidin, Orr & Roseman, 1969).

The utilization of a precursor pool of membrane subunits appears to be ruled out by several of our experiments, especially that involving long-term inhibition of protein synthesis. Recent in vivo experiments on membrane synthesis in rat liver indicate the existence of a precursor pool of membrane which is utilized over the course of 3 h following cycloheximide injection (Ray, Lieberman & Lansing, 1968). Our 6-h pre-treatment of cells should have been adequate to deplete similar precursor pools if they were present in ciiD and VA-2 cells. Furthermore, it might be expected that high energy phosphate bonds might be required for integration of any subunits into the surface, as indeed they seem to be required for initial membrane fusion in hetero-
Rapid intermixing of surface antigens

karyon formation (Okada, Murayama & Yamada, 1966). However, no inhibition of antigen spread was produced by ATP inhibitors, which in combination quickly reduced cell ATP to less than 20% of control value. This observation also tells against, though it does not rule out, mechanism iii for antigen spread. If molecules left the surface and moved through the cytoplasm before re-emerging at a new point on the surface it might be thought that their re-integration would require energy from ATP.

In arguing the last point, it may be objected that a cell containing 15–20% of its normal ATP content may well still be capable of sustaining ATP-requiring synthetic reactions. Indeed, the degree of inhibition of total ATP generation observed is proportionally far greater than the degree of inhibition of amino acid incorporation by puromycin and cycloheximide. However, this lower degree of inhibition might be expected from the work of Atkinson (1965) showing that as ADP and AMP levels rise in a cell, and ATP levels fall, substrates tend to be shifted to the Krebs cycle, generating more ATP, rather than to synthetic pathways. Despite this possibility, we must concede that inhibitor studies with DNP and NaF can only suggest, but not strongly support, the absence of a need for ATP in antigen movement.

One treatment of fused cells did inhibit the spreading and intermixing of antigens on their surfaces; this was subjection to lowered temperature. The curve of per cent mosaics formed v. temperature is that expected if spread were due to diffusion of antigen-bearing molecules in a solvent, such as lipid, whose viscosity changes markedly with temperature. The rate of spread of antigens is, assuming a value of 10 nm for the radius of an antigen molecule, consistent with a membrane viscosity of 100–200 cP(1–2 × 10^5 Ns m^-2), about that of many oils (V. A. Parsegian, personal communication). Furthermore, the curve of per cent mosaics at 40 min v. temperature is quite similar to some of those given by De Gier, Mandersloot & Van Deenen (1968) for the penetration of glycerol into liquid crystal 'liposomes' v. temperature. In each instance, the system appears to have a distinct melting temperature, above which various processes may occur.

Other natural membranes have also been shown to contain at least some fluid areas. Hubbel & McConnell (1969) used spin-labelled steroids to show that fluid regions exist in myelin and another group has recently used calorimetric techniques to indicate the liquid crystalline state of mycoplasma membranes (Steim et al. 1969). Blasie & Worthington (1969 a, b) made low angle X-ray scattering measurements of the arrangement of photopigment molecules in frog retinal receptor disk membranes, showing that the nearest neighbour frequency for those molecules was altered by change in temperature, or by the addition of antirhodopsin antibody to the system. Their interpretation of the data supports the notion that the pigment molecules 'float' in a liquid-like environment.

Our observations and calculations, and the scattered examples from the literature all call attention to the possibility that elements of many biological membranes are not rigidly held in place, but are free to re-orient relative to one another. This aspect of membrane structure has not been considered in current membrane models (cf. Stoeckenius & Engelman, 1969), though modification or extension of several of these would be sufficient to account for our results.
We are deeply grateful to Dr Hayden Coon, now of the Department of Zoology, University of Indiana, for expert advice on tissue culture techniques and cell fusion, to Dr John Cebra, for instruction in the preparation of fluorescent antibody reagents, and to Dr Jeffrey Denburg for performing the ATP assay.

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REFERENCES


Rapid intermixing of surface antigens


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Fig. 3. All cells are doubly stained with fluorescent antibodies visualizing both mouse H-2 antigens (green fluorescence) and human surface antigens (red fluorescence). Photographs were taken through filters allowing only red or green light to reach the camera. c was doubly exposed to record both colours in a single frame. For further details see the text. × 3000 approx. A. Stable somatic hybrid line MH-1. Mouse antigens are shown. b. The same. Human antigens are shown. c. $M_\frac{1}{2}-H_1$. Double exposure, for both mouse and human antigens. d. $M_1-H_1$. Mouse antigens are shown. e. $M_\frac{1}{2}-H_1$. Human antigens are shown. f. $M_1-H_1$. Mouse antigens are shown. g. $M_1-H_1$. Human antigens are shown. The plate is made from colour prints, not from original slides.
Rapid intermixing of surface antigens