Correction. In the article "Cell Cycle Changes and the Ability of Cells to Undergo Virus-Induced Fusion," by Stadler, J. K. & Adelberg, E. A., which appeared in the July 1972 issue of Proc. Nat. Acad. Sci. USA 69, 1929–1933, the following correction should be made. On pages 1932 and 1933, and in Table 3, a hybridization between mutant lines GF3 and GF7 is described. The Hgprt-deficient parent used in this cross was actually GF6, not GF3. GF6 is an independently isolated Hgprt-deficient mutant. GF3 was used for the other experiments reported in this paper, but was found to be too "leaky" for use in the hybridization experiment.
Cell Cycle Changes and the Ability of Cells to Undergo Virus-Induced Fusion
(mouse lymphocytes/cell surface/colcemid/hybrids)

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ABSTRACT Synchronized cells of the mouse leukemic cell line, L5178Y, were examined for their ability to undergo virus-induced fusion. Fusion capacity (defined as the percentage of the original unicellular population that is induced by virus to participate in a fusion event) was measured in cells that were (i) arrested by colcemid, (ii) released from colcemid block, and (iii) released from thymidine block. The results show that fusion capacity is minimal during G1 phase (less than 10%), and rises to a maximum (69%) in arrested mitosis. Treatment of nonsynchronized cells with trypsin increased their fusion capacity to the level characteristic of cells arrested in mitosis. Colcemid-arrested cells produced twice as many hybrid clones as nonsynchronized cells after they were plated in selective medium.

The surface of a mammalian cell in culture shows alterations during the cell cycle with respect to binding wheat-germ agglutinin (1) and catecholamine (2). In each case, the effective period for binding is during mitosis. The work presented here describes the relationship of certain stages of the cell cycle to the ability of the cell to participate in Sendai virus-mediated cell fusion, a process that is also dependent upon certain properties of the cell surface.

EXPERIMENTAL PROCEDURES

Cell Lines. The cell line used in these experiments was GF3, a mutant strain deficient in hypoxanthine-guanine phosphoribosyl transferase (Hgprt-), derived from a population of L5178Y (sub-clone 43) that had been treated (3) with the mutagen ICR-372. The L5178Y lymphocytic parent strain was first isolated from a leukemic mouse by Law, and later grown in vitro by Fischer (4) from whom we obtained the subclonal line 43. GF3 cells are lymphocytic in appearance, near diploid, and neoplastic.

GF3 cells are routinely grown in Fischer's medium with 10% horse serum. Spinner culture was used in all experiments, except for those involving trypsin treatment of the cells prior to viral fusion. Cell densities were determined with a model A Coulter counter.

The Sendai virus used in the fusion experiments was grown and inactivated by the method of Klebe (5).

Fusion Procedure. All lymphocyte fusions were performed in the following way: the cells were harvested at 2 to 3 × 10^6/ml, centrifuged at 1200 rpm for 10 min, taken up in chilled Earle's basic salt solution, and iced. The cell concentration was adjusted to 10^6 cells in a final volume of 1 ml. At time zero (T₀), a sample of the concentrated cells was taken for cytological examination and mixed 1:1 with lactic-aceto orcein (6), squashed lightly, and scored for cells with more than one nucleus and for the percentage of metaphase figures. The percentage of metaphase cells is the mitotic index. After the T₀ sample was taken, 2000 hemagglutinating units of Sendai virus in 0.5 ml Earle's basic salts solution and 1.0 ml of cell suspension were mixed and incubated at 4°C for 20 min. Then the mixture was transferred to a 37°C water bath, shaken gently for 3 min, and incubated without further shaking for an additional 17 min.

A second sample (T₉) was then taken for cytological examination. Random microscopic fields were scored for uninucleate and multinucleate cells. The fusion products (multinucleate) were scored for the number of included nuclei. In addition, the included nuclear material was scored as being interphase or metaphase in appearance (Fig. 1).

Fusion capacity means the percentage of single cells of the original population that have entered into a fusion product after treatment with Sendai virus. This figure is calculated by summation of the number of nuclei in the cell fusion products, subtraction of the "background" multinucleate cells present in the T₀ cytological sample (usually less than 1% binucleate

Fig. 1. Lymphocytes stained with lactic-aceto orcein after Sendai virus-induced fusion. A, fusion product containing nuclear material from 12 lymphocytes, 7 in mitosis and 5 in interphase. B, binucleate fusion product with two interphase nuclei. C, binucleate fusion product with one interphase nucleus and metaphase chromatin. D, metaphase cell damaged by virus. E, normal interphase cell.
Fig. 2. (A) Measurements of fusion capacity (FC) and mitotic index (MI) during incubation with colcemid. The doubling time of the culture at zero time was 10–11 hr. (B) The above measurements for fusion capacity plotted against those for mitotic index.

Colcemid Block. To obtain populations with large numbers of metaphase cells, log-phase cultures were grown for 4–8 hr in the presence of 25 ng/ml of colcemid (CIBA). This concentration of colcemid is not toxic to L5178Y cells, and the arrest is completely reversed by removal of the colcemid (7). Increases in the number of metaphase cells were determined at hourly intervals. In all fusions performed with colcemid-blocked cells, colcemid was retained in the virus–cell mixture at a final concentration of 25 ng/ml.

Thymidine Block. A single thymidine block was used to synchronize populations by arrest in early S phase. The cells were grown for 5 hr in the presence of 2.5 mM thymidine, then released by washing and resuspension in warm medium containing 1 μM deoxycytidine (7). The degree of synchrony obtained was monitored by measurement of subsequent growth and increase in mitotic index.

RESULTS

The effect of colcemid arrest on fusion capacity

GF3 ordinarily grows with a 10- to 11-hr doubling time. Fig. 2A presents the results of an experiment in which fusion capacity is followed as the culture is incubated with 25 ng/ml of colcemid, and as the proportion of arrested metaphase cells increases with time. The first fusion experiment was performed on cells removed from the culture just before the addition of colcemid. Subsequent fusions were done with samples taken at 1, 4, and 8 hr after the addition of colcemid. It is clear that both fusion capacity (with a base-line figure of 20%) and mitotic index increase linearly with time, but that the mitotic index increases faster than does fusion capacity.

When these slopes are plotted against each other (Fig. 2B), the resultant line has a 60% slope; i.e., the chance of a cell’s entering into a successful fusion product increases by 60% as the culture accumulates arrested metaphase cells. In control experiments, cells grown without colcemid were fused with Sendai virus both in the presence and absence of colcemid; the colcemid added to the fusion mixture had no effect on fusion capacity. Thus, the stimulation of fusion described above depends on the growth of cells in the presence of colcemid.

The observed stimulation could be accounted for in one of

Table 1. Chi square analysis of the distribution of metaphase and interphase nuclei in the binucleate fusion products of GF3 populations growing with a 10- to 11-hr doubling time

<table>
<thead>
<tr>
<th>Hr after addition of colcemid</th>
<th>Observed frequencies of metaphase (m) and interphase (i) single cells</th>
<th>Expected class frequencies</th>
<th>Expected value (E)</th>
<th>Observed value (O)</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>m = 0.06 i = 0.94</td>
<td>i² = 0.8836</td>
<td>23.8</td>
<td>19</td>
<td>8.309</td>
<td>0.01-0.02</td>
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<td></td>
<td></td>
<td>het = 0.1128</td>
<td>3.05</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m² = 0.0036</td>
<td>0.00002</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>m = 0.07 i = 0.93</td>
<td>i² = 0.8649</td>
<td>38.05</td>
<td>31</td>
<td>10.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>het = 0.1302</td>
<td>5.72</td>
<td>13</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>m² = 0.0049</td>
<td>0.23</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>m = 0.26 i = 0.74</td>
<td>i² = 0.5476</td>
<td>36.14</td>
<td>28</td>
<td>11.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>het = 0.3848</td>
<td>25.4</td>
<td>27</td>
<td>11.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>m² = 0.0676</td>
<td>4.4</td>
<td>11</td>
<td>0</td>
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</tr>
<tr>
<td>8</td>
<td>m = 0.50 i = 0.50</td>
<td>i² = 0.25</td>
<td>20</td>
<td>22</td>
<td>8.05</td>
<td>0.01-0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>het = 0.50</td>
<td>40</td>
<td>48</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>m² = 0.25</td>
<td>20</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
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</table>

m = the frequency of observed metaphase cells in the population at each time point and i = the frequency of interphase cells in the same population. The expected class frequencies are calculated from (m + i)² = 1. Chi square is computed by the formula χ² = Σ(O−E)²/E. P values have been determined with two degrees of freedom.
two ways: (i) metaphase cells have a higher probability of fusion than do interphase cells; or (ii) colcemid exerts a time-dependent effect on growing cells that increases their fusion capacity by a mechanism unrelated to their stage in the cell cycle.

It is possible to discriminate between these alternatives by a simple statistical analysis of the binucleate fusion products from each time point of Fig. 2A. This analysis is presented in Table 1. If a cell's fusion ability is distributed randomly among all cells of both the arrested metaphase population and the interphase population, then the distribution of these cell types among the binucleate fusion products should approximate the distribution predicted by the expansion of \((m + i)^2 = 1\), where \(m\) is assigned the frequency of arrested metaphase cells and \(i\) the frequency of interphase cells. The \(\chi^2\) values at the three early time points tell us that the distributions have one (or less than one) chance in a hundred of being random. In each case, the nonrandomness observed is due to an excess of fusion products containing the nuclear material of at least one metaphase cell.

The nonrandom distribution at the 8-hr point is due to fewer than expected fusions of two metaphase cells. Cytological examination of single metaphase cells at this time showed that many suffer great damage after viral treatment and exist as naked masses of nuclear material (Fig. 1). These vulnerable cells may represent a class of the metaphase population that has been in colcemid arrest for as long as 8 hr, and is unable to repair their cell membrane after viral attack.

From the preceding analysis, it is clear that metaphase cells are preferentially involved in Sendai virus-induced fusion, whether they occur spontaneously (0 hr, Table 1), or are retained for a short time by colcemid arrest (1 hr and 4 hr, Table 1). It is interesting that the increased fusion capability of these cells involves them in fusions with interphase cells until their frequency is high enough (mitotic index = 20%, 4 hr) that they preferentially fuse with other metaphase cells.

Experiments with fast-growing cells

In working with GF3 cells, we have found that cells in spinner culture will grow with a 6- to 8-hr generation time if medium is added at daily intervals. A study of fusion capacity during colcemid blockade with these fast-growing cultures gives us additional information about the capacity for viral fusion at specific times during the growth cycle.

Some data for fast-growing cells are presented in Fig. 3A, where mitotic index and fusion capacity are plotted against duration of exposure to colcemid. These results can be summarized as follows: (a) at 0 hr, there is a greatly increased baseline fusion capacity (40% as against 20% for the slower-growing cultures); (b) there is a linear increase in mitotic index to 50% in 5 hr (as opposed to 8 hr for the slower-growing culture); and (c) there is an overall increase in fusion capacity of 75%. However, this increase in fusion capacity is nonlinear.

In Fig. 3B, where fusion capacity is plotted directly against mitotic index, the linear relationship evident for slow-growing cells (Fig. 2B) is absent; instead, the curve of best fit has two slopes. The second slope (B–C, Fig. 3B) parallels that obtained with slow-growing cells. However, the early slope, between 0 and 1 hr (A–B), shows that the percent of single cells involved in fusions increases almost twice as rapidly as the mitotic index, i.e., an 8% rise in metaphase cells and a 15% increase in fusion products. Clearly, the increase in number of metaphase cells cannot account for the entire increase in fusion capacity. This finding raises the question as to what other class of cells might be fusing with such great efficiency at early times.

Studies of synchronized L5178Y populations growing at a 10-hr generation time have led to the following estimates of time involved for each stage of the cell cycle: mitosis, 0.5–1 hr; G1, 1–2 hr; S, 6–7 hr; G2, 1 hr (8, 9). In the present experiments, where the entire population grows with a shorter doubling time, it would be expected that within 1 hr after colcemid blockade the proportion of G1 cells in the population should be near zero. Cells entering G1 at the time of addition of colcemid would by 1 hr have progressed into S phase, and the mitotic cells would have been prevented from entering G1. Conversely, the proportion of S and G2 cells should, by 1 hr, be increased relative to their proportion in a nonsynchronized population. This reasoning leads us to believe that the interphase cells that participate actively in fusions at 1 hr cannot be in G1, but must be in either S or G2.

Table 2 presents the \(\chi^2\) analysis of the distribution of interphase and metaphase nuclear material in binucleate fusion products at each fusion time of Fig. 3A. In contrast to the results of this type of analysis of fusion products of slow-growing cells (Table 1), it is only the two earliest points that show unequivocal nonrandom involvement of metaphase cells in fusion. At the later 2.5- and 4.5-hr points, there is a one in ten chance that the distribution is random. Increasing randomness correlates well with the observation that fusion capacity does not rise as quickly as mitotic index during the latest time interval measured. This does not reflect a general deterioration.
of the cells arrested for 5 or 8 hr, since the population shows no more than a 1% loss in viability (as measured by trypan blue exclusion) and growth is resumed quickly after release from colcemid.

We interpret the latter observations above to mean that the physiological state of the metaphase cell that renders it most capable of fusion disappears gradually as the cells are incubated with colcemid.

The fusion capacity of cells in interphase

Because of the suggestion that G1 cells have a low fusion capacity, two additional types of experiments were performed in order to obtain more data pertaining to the fusion capability of cells in different stages of interphase. In the first series of experiments, fusions were performed with populations of cells released from a single thymidine block and, in the second, fusion capacity was followed in populations released from colcemid block.

Fig. 4 presents the fusion capacities and mitotic indexes obtained in one such thymidine block and rel ease experiment.

1 hr after release, when a relatively large proportion of the cells should be in S phase (7), the mitotic index drops by half and the fusion capacity is greatly depressed. 6 hr after release there is a small "mitotic wave" and fusion capacity improves, but does not approach that of nonsynchronized cultures. These data, though not extensive, indicate that cells in early S phase do not fuse well.

Fusion capacities and mitotic index at various time points after release from colcemid block are given in Fig. 5. In the first 0.5 hr, the fusion capacity drops as quickly as the mitotic index drops. It is striking that at 1 hr after release, the fusion capacity falls much below that in nonsynchronized populations. During the first hour, fusion capacity is inversely proportional to the percentage of clearly recognizable G1 cells in the culture.

The effect of trypsin on the fusion capacity of nonsynchronized cells

Cells of the nonmalignant mouse line 3T3 bind wheat germ agglutinin during mitosis, but not during interphase (1). Interphase cells, however, become agglutinable by this lectin if they are treated with low concentrations of trypsin (10). These results suggest a similarity between the surfaces of mitotic cells and the surfaces of trypsin-treated interphase cells; accordingly, we have asked whether trypsin affects the fusion capacity of nonsynchronized (mainly interphase) cells.

Log-phase cells of GF3 were washed, resuspended in a solution containing 0.01% trypsin in Earle's basic salts solution, and incubated at 37°C for 6 min. The treatment was stopped by the addition of a 20-fold excess of soybean inhibitor, and the cells were resuspended in 1.0 ml of warm medium (10). 2000 Hemagglutinating units of Sendai virus were then added, and cells were fused. Trypsin treatment increased the fusion capacity of the culture from 27 to 63%.

The formation of genetic hybrids by colcemid-arrested cells

Two mutant lines have been derived from L5178Y in this laboratory: GF3 (Hgprt deficient) and GF7 (thymidine kinase
Table 3. The number of hybrid clones derived from colcemid-treated and untreated parent strains

|                  | Total number of revertant clones observed | Total number of hybrid clones observed | Number of hybrid clones per 10^6 cells plated |
|------------------|------------------------------------------|---------------------------------------|
| GF3              | 4 × 10^6                                  | 0                                     | 0                                               |
| GF7              | 4 × 10^6                                  | 0                                     | 0                                               |
| GF3 x GF7        | 82 × 10^6                                 | 545                                   | 6.6                                             |
| (colcemid-       |                                          |                                       |                                                 |
| treated)         |                                          |                                       |                                                 |
| GF3 x GF7        | 48 × 10^6                                 | 151                                   | 3.1                                             |
| (untreated)      |                                          |                                       |                                                 |

The parental and virus-treated cells were plated in a series of 16 × 125 mm screw-cap tubes, each containing 5 ml of selective medium and 0.12% agar (11). 2 × 10^6 cells were plated per tube.

(Tk) deficient. Cells of GF3 and GF7 were mixed in a ratio of 1:1 to make a total of 10^6 cells in 1.0 ml. They were then fused with 2000 hemagglutinating units of Sendai virus. Immediately after fusion, the entire mixture was diluted and cloned in agar medium (11) containing hypoxanthine, aminopterin, thymidine, and glycine, which is selective for Hgprt+, Tk+ hybrids (12). Each parent cell type was plated individually as a control. Hybrid clones were visible at 7 days and counted at 10 days; each clone contained at least 10^4 cells.

The results are shown in Table 3; the colcemid-treated parent cultures produced twice as many hybrid clones as the untreated controls, roughly paralleling their increase in fusion capacity.

**DISCUSSION**

Virus-mediated cell fusion is a complex process. In general terms, it can be considered to occur in three steps: an initial step of viral adsorption, an intermediate step involving a change in the organization of the cell coat and/or membrane, and the final step of cell membrane fusion. The condition of the membrane that is produced by the intermediate step, and that triggers fusion, is not understood. Lucy postulates that the critical condition is a shift from the bimolecular leaflet state to the micellar state of the membrane phospholipids (13); Poste postulates that the critical condition is a thinning of the cell coat so that cell-to-cell contact in the primary minimum (10 Å) may be achieved, and that this close contact itself initiates membrane fusion (14).

In view of our own experimental results, it is of interest that Bosmann and Winston have reported cyclical changes in the synthesis and excretion of glycolipids and glycoproteins by synchronized cells of L5178Y (8). For both classes of macromolecules, excretion is at a minimum in G1 and S, and at a maximum in late G2 and M. In the case of glycoproteins, excretion is preceded in the cell cycle by synthesis, which is maximal during S; in the case of glycolipids, excretion and synthesis are simultaneous.

The parallel between fusion capacity and the excretion of glycoproteins and glycolipids is striking, and suggests that fusion capacity is dependent on an optimum concentration of one or both of these classes of macromolecules at the cell surface. The finding that treatment with trypsin raises the fusion capacity of interphase cells to the high level characteristic of colcemid-blocked mitotic cells is compatible with the view that the concentration of surface glycoproteins is critical for the fusion process.

We thank Mrs. Tara Kasturi for her technical assistance and Dr. Martine Armstrong for determining the malignancy of GF3. This work is supported by Grant GB22864 from the National Science Foundation.