MOUSE LEUKEMIA VIRUS: "SPONTANEOUS"
RELEASE BY MOUSE EMBRYO CELLS AFTER LONG-TERM IN VITRO CULTIVATION*

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Abstract.—BALB/c mouse embryo cells maintained in tissue culture on a schedule of rapid transfer at high cell density develop into tumorigenic lines that have lost contact inhibition of cell division. After several months in culture, certain of these lines begin to release mouse leukemia virus. This virus has properties indistinguishable from those of virus found in adult BALB/c mice. The evidence presented here demonstrates that the murine leukemia virus genome must be present in the original embryo cultures. The possibility that the genetic information for making murine leukemia virus is present in a repressed form in every mouse embryo cell is discussed.

Introduction.—In 1962, several established lines were developed from one pool of random-bred Swiss mouse embryo cells. The cell lines that emerged differed markedly from one another with regard to their degree of contact inhibition of cell division. The 3T3 line (transferred every three days and always reinoculated at $3 \times 10^6$ cells/plate) retained an exceptionally high degree of contact inhibition. Lines 3T6 and 3T12 from the same embryo culture, maintained in parallel with 3T3 but always transferred at higher cell densities ($6 \times 10^6$ and $12 \times 10^6$ cells/plate, respectively), lost the property of contact inhibition and became able to form multiple cell layers under culture conditions where the 3T3 cells were restricted to a monolayer.

The same culture conditions and transfer schedule were used more recently to develop lines from inbred BALB/c mouse embryo cultures. The BALB/3T3 lines are highly sensitive to contact inhibition while the BALB/3T12 lines have lost this property. The latter, but not the former, have also acquired the ability to produce tumors when inoculated into newborn or irradiated weanling BALB/c mice.

In the report describing the tumorigenic properties of the BALB/3T3 and BALB/3T12 cell lines it was noted that one line of BALB/3T12, began to release mouse leukemia virus (MuLV) after many cell generations in culture although the original embryo culture and earlier transfer generations of this BALB/3T12 line were negative for virus. In the present study we have analyzed the cell lines derived from the BALB/c embryo culture and the original Swiss embryo culture for the production of mouse leukemia virus antigen and infectious murine leukemia virus. Several independently maintained cell lines were found to begin producing murine leukemia virus after many months in culture, and the data to be presented strongly suggest that the virus and/or the capacity for virus production is present in the original embryo cells. Certain cell culture procedures favor the
appearance of viral antigen and infectious virus, while other conditions prevent or retard their appearance.

Methods.—Cell cultures: Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.) was used for growing all cells. The cells were maintained in 50-mm plastic Petri dishes and were subcultured using 0.1% trypsin in phosphate buffered saline. The development and characterization of the Swiss embryo cell lines and the BALB/c embryo cell lines have been described in detail elsewhere. With both sets, cells were always reinoculated at a constant number (see below), regardless of their growth rate between transfers. In the initial Swiss embryo series, both three-day and six-day transfer schedules were employed; with the BALB/c embryo cells only three-day transfers were used. In each case the same growth pattern was observed. The cells grew rapidly for 5-10 transfers, then quite slowly for the next 10-30 transfers. With proper care, however, cells emerged that once again could grow rapidly.

The terms 3T3, 3T12, 6T6, etc., define the conditions of transfer used to develop the lines. The first number indicates the interval between transfers in days, the second shows the number of cells inoculated (× 10⁶) per plate. Where more than one line was developed using a single transfer scheme, they were distinguished from one another by a third number, e.g., 3T12-1 and 3T12-2. The BALB/c embryo cell lines were designated in the same manner but with the addition of the prefix, BALB, e.g., BALB/3T12-1.

Detection of murine leukemia virus group specific antigen: Cell extracts were prepared from confluent cultures as 20% (v/v) suspensions in supernatant fluid. A portion of the extract was frozen and thawed once and sonicated for 2-3 sec with a Branson sonifier with a microtip attachment. The sonicates were titered for complement-fixing (CF) antigen of the murine leukemia-sarcoma virus group using rat antiserum to a moloney murine sarcoma virus (M-MSV) induced tumor. In order to eliminate anticomplementary activity due to calf serum used in the growth medium, cultures were washed once and changed to medium containing 10% fetal bovine serum 2-3 days before harvesting.

Virus isolation and characterization: Cell extracts prepared as above were frozen and thawed once, aspirated with a fine needle and syringe, and centrifuged at 2000 rpm for 10 min to remove cell debris. Presence of murine leukemia virus was determined by inoculation of the extracts onto secondary mouse embryo cultures prepared from NIH strain Swiss or BALB/c mice and testing for induction of CF antigen (comul test) or fluorescent antibody (FA) stainable antigen. For fluorescent antibody testing, coverslip preparations of cultures inoculated with cell extracts were fixed at seven days in cold acetone and stained by the indirect procedure using M-MSV rat serum and fluorescein-conjugated goat anti-rat gamma globulin (prepared by Dr. R. Wilsnack, Baltimore Biological Laboratories, Baltimore, Md.). All negative virus isolation harvests were blind passaged at least once.

For virus neutralization tests, the antisera used included the following: M-MSV rat serum which neutralizes murine leukemia virus of the FMR subgroup but not the Gross subgroup; Gross passage A rat serum; and AKR rat serum (obtained from Fischer rats carrying transplantable lymphoma induced by a tissue culture isolate from a spontaneous AKR strain leukemia); the latter two sera neutralize all the strains of naturally occurring mouse leukemia virus so far tested.

Results.—Mouse leukemia virus release by mouse cell lines: When the various BALB/c cell lines were screened for murine leukemia virus complement-fixing antigen in the course of tumorigenicity studies one line, BALB/3T12-4, was found to be positive. For this reason all the cell lines of both the BALB/c and the Swiss embryo cell series were studied. The results of the CF tests are shown in Table 1. The original BALB/c embryo culture from which all the BALB lines were derived was negative by CF and was also negative for virus isolation by the comul test. Many other BALB/c embryo cultures have been tested for murine leukemia virus, and all have been found to be negative. BALB/3T3
was also negative and has remained negative for murine leukemia virus CF antigen through more than 200 cell generations in culture. In the Swiss embryo series, two out of five separately carried lines were found to be positive for complement-fixing antigen though the original embryo culture again was negative; the two Swiss lines that were positive, 3T12-2 and 6T6, were both lines that had lost contact inhibition and now had high saturation densities in culture (>5 × 10^6 cells/plate).

During the establishment of the BALB/c cell lines, cultures had been frozen down at various passage levels. Thus the BALB/3T12-4 line could be re-examined for appearance of viral antigen and infectious virus starting from an early passage. Figure 1 shows that up to the 30th passage level, BALB/3T12-4 was negative for CF antigen. Shortly thereafter, viral antigen was first detected and by the 50th generation the line was positive by CF at a titer of 1:16. Even as late as the 25th transfer (75 days), the cells were negative for antigen by the CF test, and by a comul test involving two blind passages in NIH Swiss embryo
indicator cells. One of the antigen-positive Swiss lines, 3T12-2, was negative for viral antigen at 30 cell generations. By 100 cell generations, however, it was producing not only viral antigen but also infectious murine leukemia virus.

At the 25th passage, BALB/3T12-4 was studied for the presence of viral antigen by fluorescent antibody staining. Out of three cover slip preparations, each of which contained about $2 \times 10^6$ cells, one small positive focus was found. This was the first evidence of viral-antigen production in the line. By the 35th passage, 0.1 percent of the cells were positive by fluorescent antibody though the culture was still negative by the CF test (Table 2).

<table>
<thead>
<tr>
<th>Generations in culture</th>
<th>Fluorescent antibody (% cells positive)</th>
<th>MuLV CF titer (reciprocal)</th>
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<tr>
<td>25</td>
<td>0.001</td>
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<td>35</td>
<td>0.1</td>
<td>&lt;1</td>
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<tr>
<td>40</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50</td>
<td>&gt;50</td>
<td>16</td>
</tr>
<tr>
<td>200</td>
<td>&gt;50</td>
<td>&gt;32</td>
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A portion of cells at the 40th passage level were tested for viral antigen by fluorescent antibody staining while others were simultaneously plated at low cell numbers for cloning or begun on a schedule of sparse (3T3) or dense (3T12) passage. Ten clones were selected and grown for CF testing (approximately 25 additional cell generations). The sublines on either a 3T3 or 3T12 transfer schedule were serially passaged for the same period of time (10 transfers). Of the clones selected, one out of ten was found to be positive for viral antigen, approximately the same fraction that was fluorescent antibody positive in the initial population. However, the sublines carried on either a 3T3 or a 3T12 transfer schedule were both highly positive, with titers greater than 1:32. Thus, cloning of cells prevented the subsequent increase in the percentage of virus-positive cells. The mass cultures at sparse and dense passage could have become highly positive either because of the spread of virus to uninfected cells, because of a selective growth advantage of the virus containing cells, or because conditions were now “right” for the viral information to be expressed. The possible selective growth advantage of the virus-producing cells was tested by comparing growth rates and saturation densities of negative and positive clones of BALB/3T12-4. Both the virus-negative and the virus-positive clones were found to have essentially the same growth rates and saturation densities. In terms of their tissue culture behavior, it was not possible to recognize differences between the virus-producing clones and the nonproducing clones.

To test the possibility that a very small amount of virus could be present in the original embryo culture and still require over 30 passages to grow out in BALB/3T12-4 under its conditions of passage, a reconstruction experiment was performed; a portion of the original BALB/c embryo culture was started again on a “3T12” transfer schedule with and without the addition of approximately 10–50 units of infectious virus obtained from BALB/3T12-4 (see below). Table 3 shows that after six transfers, both the control and virus-infected cultures were
negative by CF for viral antigen. However, after 12 transfers (36 days after exposure) the virus infected embryo culture was positive at a titer of 1:8. In the original line that "spontaneously" released virus there was no detectable CF activity for over 35 transfers and well over 100 days in culture. A titer of 1:16 was not achieved until 50 cell generations (approximately 150 days). The reconstruction experiment shows that if a very low level of infectious virus had been present in the initial embryo cells, the method of passaging the cells should have allowed it to appear much sooner than it did appear in BALB/3T12-4.

Properties of the "recovered" viruses.—The host range of the virus released by BALB/3T12-4 was compared in NIH Swiss and BALB/c secondary mouse embryo cultures by measuring the relative ability of the preparations to induce fluorescent antibody stainable murine leukemia virus antigen (Table 4). At seven days, the cover slips were stained by the fluorescent-antibody method for viral antigen. Infected NIH Swiss embryo cultures exhibited typical cytoplasmic staining sparing the nucleus in approximately 10 per cent of the cells while cover slips containing BALB/c embryo showed only about 0.5 per cent fluorescent antibody positive cells (Table 4). Virus released by BALB/3T12-4 at 50 generations and 200 generations and by cultured cells from tumors induced by BALB/3T12-4 all showed the same relative growth ability in NIH Swiss and BALB/c cells; there was no evidence even on continuous growth in BALB/c cells of adaptation of the virus to the BALB/c cell. The CF antigen titers attained in the NIH Swiss cells as compared to BALB/c cells also indicated the greater sensitivity of the NIH Swiss system for growth of virus released from BALB/3T12-4. Of note is the fact that the murine leukemia virus group viruses isolated from a high percentage of adult and geriatric BALB/c mice show this

<table>
<thead>
<tr>
<th>Table 4. Growth properties of virus released from two long-term mouse 3T12 lines.</th>
<th>Embryo Test Cells</th>
<th>NIH</th>
<th>% FA</th>
<th>CF</th>
<th>% FA</th>
<th>BALB/c</th>
<th>CF</th>
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<tbody>
<tr>
<td>Source of virus</td>
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<tr>
<td>BALB/3T12-4</td>
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<td>200 generations</td>
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<td>Tumor cells</td>
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<td>Late passage†</td>
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<td>Swiss/3T12-2</td>
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<tr>
<td>10</td>
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<td>4</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>10</td>
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<td>0.2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2</td>
<td>4</td>
<td>0.1</td>
<td>&lt;1</td>
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<tr>
<td>10</td>
<td>16</td>
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*Approximate per cent of cells with FA-stainable antigen at 7 days.
† Reciprocal of CF antigen titer of 3-week harvest.
‡ Tumor cell isolate tested after three passages in NIH Swiss mouse embryo cells.
same pattern of host susceptibility; the majority grow well in NIH Swiss embryo cells but relatively poorly in BALB/c embryo cells.8

Neutralization studies further characterized the BALB/3T12-4 virus as typical of the naturally occurring leukemia viruses found in adult BALB/c mice. Infection of NIH Swiss cultures with BALB/3T12-4 virus, as detected by induction of fluorescent antibody-stainable antigen, could be inhibited by antisera to Gross passage A virus and an isolate from spontaneous AKR leukemia but could not be inhibited by antisera directed against viruses of the FMR group.

The virus recovered from the Swiss/3T12-2 line showed a similar host range specificity (Table 4); it was isolated using NIH Swiss embryo cells. The host range was similar to that of the majority of naturally occurring murine leukemia virus isolates from inbred and random bred strains.8 This Swiss/3T12-2 isolate is of interest since two other Swiss colonies (NIH and SWR/J) have been intensively studied and, although murine leukemia virus group specific CF antigen has been detected occasionally in leukemic or normal lymphoid tissue, the virus has not yet been grown in cell culture.8 Nothing, however, is known of the frequency of age distribution of naturally occurring murine leukemia virus in the mouse colony from which the original Swiss 3T3 and 3T12 cultures were derived.

Discussion.—Among the possible explanations for the “spontaneous” appearance of mouse leukemia viruses in mouse cell cultures, the first that must be considered is accidental laboratory contamination. We feel this is very unlikely for the following reasons: (1) This work was done at a time when no leukemia viruses had ever been used in either the laboratory in New York or the one at NIH, and at neither place were mouse colonies maintained. (2) The virus that does emerge from the cell cultures is not typical of laboratory murine leukemia virus, such as Friend, Moloney, and Rauscher virus,6 but, rather, is a virus with properties very similar to those of the virus which “spontaneously” appears in nontumorous adult BALB/c mice.8 One of the characteristics of this virus is that it grows very poorly in BALB/c embryo cultures—the cells from which it emerges. (3) Finally, while virus has been obtained from certain lines, others, maintained in the same incubator on the same transfer schedule, have not shown in repeated tests, any evidence of virus.

If the appearance of infectious virus is not the result of exogenous infection, then the viral genome must be present in the original primary mouse embryo cells. The probability of virus expression is relatively low. Only 3 out of 11 independently maintained cell lines so far have been shown spontaneously to release virus. All three of these were lines where the cells were transferred frequently and always at high cell densities. Rapidly dividing, DNA-synthesizing cells are known to favor the replication of mouse leukemia-sarcoma virus.10 Crowded culture conditions with extensive cell–cell contact, may have favored the emergence of virus by facilitating, for example, the transfer of information from cell to cell.

There remain, then, two possibilities for the spontaneous release of virus from mouse embryo cells. One possibility is that there is a very low level of infectious virus in the initial embryo culture that cannot be detected by conventional
methods. The other is that many, and perhaps all, mouse cells contain, in a repressed form, the information to make mouse leukemia virus. The first possibility is unlikely since after 25 transfers, less than one cell in $10^6$ was producing viral antigen. The reconstruction experiment showed that 10–50 infectious virus particles from BALB/3T12-4 were able to grow out to high titer within 12 transfers when added back to the original embryo cells. If a very low level of infectious virus had been present initially in the embryo cultures, we can conclude that it should have appeared at a much earlier time. The experiments described above, then, are consistent with the hypothesis that the mouse cells contain the information needed to make mouse leukemia virus.

Huebner has discussed the evidence that most naturally occurring tumors of mice appear to be caused by viruses which are members of the murine leukemia-sarcoma complex. The evidence indicates that the virus genome can be transmitted vertically through the ovum. Expression of virus and/or oncogenic potential are controlled by the genetic makeup of the host and are influenced by environmental factors, such as exposure to radiation and chemical carcinogens. The information for making infectious virus remains almost totally repressed in at least some strains of Swiss mice, and only begins to appear after 4–6 months of age in the spleens of BALB/c strain mice. Treating BALB/c mice with agents such as the chemical carcinogen methylcholanthrene results in virus antigen production at a much earlier time and with a higher incidence than in untreated animals. The incidence of tumors rises concordantly. In a similar manner, Gross and Lieberman, et al. have shown that radiation-induced leukemia in C57B1 mice is associated with the appearance of mouse leukemia virus in the tumor cells.

The induction of virus from mass cultures of mouse embryo cells resembles at least superficially the induction of prophage from lysogenized bacteria; in both cases, there is a certain low probability of spontaneous virus production. In the latter, the efficiency of induction can be greatly enhanced by chemical mutagens and radiation.

All of the above supports the hypothesis of Huebner and Todaro that the C-type RNA-virus information is able to maintain a cellular location and be transmitted vertically both from animal to progeny animal and from cell to progeny cell without being expressed as infectious virus. The cells may be able to inhibit the expression of viral functions perhaps in a manner similar to the repression of bacteriophage functions and to the possible repressor activity of oncogenic DNA viruses.

Direct proof of this hypothesis would require that specific manipulations in tissue culture of a virus negative line derived from a single cell would result in the release of infectious virus. Agents such as radiation and chemical carcinogens as well as methods of increasing cell–cell interaction in culture are currently being tested.

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† Viral Carcinogenesis Branch, National Cancer Institute.
† Laboratory of Viral Oncology, National Institute of Allergy and Infectious Diseases.

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