In Vitro Induction of Continuous Acute Promyelocytic Leukemia Cell Lines by Friend or Abelson Murine Leukemia Virus

By Joel S. Greenberger, Patricia B. Davisson, Paula J. Gans, and William C. Moloney

Long-term adult NIH/Swiss mouse bone marrow cultures (2.0 × 10^5 cells/10 ml Corning flask, Fisher's medium, 25% horse serum, 10^-7 M hydrocortisone) were infected with RNA type-C viruses at wk 4, and weekly removed cells were assayed for number, morphology, granulocyte-macrophage progenitor cells (CFUs), pluripotent hemopoietic stem cells (CFUs), and growth in liquid culture in 10% WEHI-3 CSF. Cultures infected with Friend leukemia virus, anemia-inducing strain (FLV-A), showed increased CFU 3 wk after virus addition, and removed nonadherent cells from 40% of cultures grew as continuous lines. Each of 12 0.8% methyl-cellulose-cloned sublines from one line (FLV-A 16-IV) showed CSF independence, promyelocyte morphology, and synthesis of esterase-M (ASD-chloroaacetate substrate), myeloperoxidase, and lysozyme (2.0-10.0 μg/10^6 cells/24 hr); each contained 2%-10% cells with mature granulocyte morphology; and each caused acute myelogenous leukemia in adult mice. Four of eight cultures inoculated with Abelson murine leukemia virus (A-MuLV) with Moloney-MuLV helper virus coat produced CSF-independent basophilic promyelocytic leukemia lines by 5 wk after addition of virus. In contrast, cultures infected with long-latent-period leukemogenic viruses (BALB: virus-1 or Rauscher-MuLV) produced increased numbers of immature granulocytic cells for 20 wk, which, although not establishing as continuous lines, were morphologically dysplastic as compared with control uninfected cultures. Distinguishable grades of virus-specific granulocyte transformation were detected in corticosteroid-treated long-term marrow culture.

Since their discovery in the 1950s the murine type-C RNA leukemia viruses (retroviruses) have been intensively studied as model etiologic agents for human leukemia. The biochemical mechanism for replication and integration of viral genetic sequences into host cell DNA has been partially elucidated, and the proviral DNA sequences for multiple copies of leukemogenic viral genomes have been detected by sensitive molecular biologic techniques in the DNA of mouse embryo cells. Both virus-specific and host-specific genetic factors are known to be very important for leukemogenesis in vivo. The recent discovery of vertically transmitted (“inherited”) viral genomes that can be induced to produce a distinct class of xenotropic viruses incapable of growth in host cells has uncovered new variables for analysis of why some viruses cause leukemia and others do not.

In vivo studies of the cellular biology of virus-induced leukemias have been complicated by several variables: the host’s immune response to the virus, which may contribute to or even provide the target cells for transformation; the presence of multiple hematopoietic organs containing potential target cells for transforma-
tion;\textsuperscript{18-12} the presence in these hematopoietic organs of cells containing other endogenous RNA leukemia viruses that may interact\textsuperscript{13,14} and genetically recombine\textsuperscript{15} with the infecting virus. Studies with Swiss mice, a strain containing no interfering ecotropic endogenous virus,\textsuperscript{5,16} have aided in controlling for the latter variable; however, the former two variables can be eliminated only through use of an in vitro system. Such a system has recently been developed by Dexter and associates,\textsuperscript{17} and studies showing morphologic and functional alterations of bone marrow granulocytic cells by an added polycythemia-inducing strain of Friend virus (FLV-P) have recently been published.\textsuperscript{18} We now report in vitro induction of stably transformed cell lines of leukemogenic promyelocytes in bone marrow cultures infected either by another strain of Friend virus (FLV-A) or by Abelson murine leukemia virus.

\textbf{MATERIALS AND METHODS}

\textbf{Mice}

NIH/Swiss mice were obtained from the animal colonies of the National Institutes of Health.

\textbf{Tissue Culture}

Cell lines included NIH/3T3,\textsuperscript{19} BALB/3T3,\textsuperscript{20} and NRK.\textsuperscript{21} A nonproducer clone of each transformed by a Kirsten murine sarcoma virus (KiMSV) has been reported,\textsuperscript{22} and these are designated K-NIH, K-BALB, and K-NRK, respectively. Cells were grown in Dulbecco’s modified Eagle (DME) medium in 10% fetal calf serum (Colorado) in plastic petri dishes (Falcon Plastics, Los Angeles, Calif). A clonal subline of the WEHI-3 BALB/c mouse monomyelocytic leukemia,\textsuperscript{23} designated the WEHI-3 clone 3,\textsuperscript{24} was grown in RPMI-1640 medium with 10% fetal calf serum; 72-hr supernatants were concentrated fivefold by Amicon filtration according to published procedures,\textsuperscript{25} and this was added to Alpha medium (GIBCO) in a 10% volume as WEHI-3 CSF.

\textbf{Viruses and Virus Assays}

Friend leukemia virus, anemia-inducing strain (FLV-A), was generously provided by Dr. Charlotte Friend, Mt. Sinai Hospital, New York, N.Y. This stock virus was passaged to a single litter of newborn NIH/Swiss mice, and peripheral blood erythroleukemia and splenomegaly uniformly developed by 28 days. Spleens from leukemic mice were explanted, single-cell suspensions were prepared, and cells were incubated for 24 hr in DME medium with 10% fetal calf serum. This medium was then filtered through 0.2-μm filters (Naglene), and virus stocks were frozen at \(-85\) °C. A stock of Abelson leukemia virus with the Moloney-MuLV helper virus coat, designated A-MuLV(M-MuLV), was generously provided by Drs. Naomi Rosenberg and Alan Silverstone, Massachussetts Institute of Technology, Boston, Mass. A clonal strain of Rauscher leukemia virus designated M52R has previously been reported.\textsuperscript{26} This virus causes only lymphoid leukemia in newborn NIH/Swiss mice, with a mean latent period of 30 wk.\textsuperscript{27} A stock of the endogenous virus of BALB/c mice designated BALB:virus-1 has been reported.\textsuperscript{27} This virus causes chronic myeloid leukemia in newborn NIH/Swiss mice, with a mean latent period of 48 wk.\textsuperscript{27}

Viruses were standardized by reverse transcriptase assay of serial 10-fold dilutions titered onto NIH/3T3 cells to 10\textsuperscript{9} polymerase induction units (PIU) per milliliter according to published procedures\textsuperscript{48} and also by XC plaque-forming assay.\textsuperscript{41} Initial virus titers were 10\textsuperscript{6} PIU from FLV-A leukemic spleen supernatants titered onto NIH/3T3 cells and 10\textsuperscript{5} PIU for stock A-MuLV(M-MuLV). The R-MuLV designated M52R and BALB:virus-1 titers in NIH/3T3 were 10\textsuperscript{6} and 10\textsuperscript{5} PIU, respectively, prior to concentration. Virus was diluted in serum-free Dulbecco’s modified Eagle medium or concentrated by ultracentrifugation at 100,000 g for 30 min and resuspended at 10\textsuperscript{5} PIU in 0.1 ml of medium.

\textbf{Virion-Associated Reverse Transcriptase Assay}

The medium of the virus-producing cultures was changed, and 24 hr later supernatants were harvested and assayed for RNA-dependent DNA polymerase activity after 100-fold concentration. Briefly, reaction mixtures were incubated at 37°C for 60 min and contained in 0.05 ml: 0.05-M Tris
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HCl, pH 7.8; 0.06-M potassium chloride; 0.002-M DTT; 2 × 10^{-4}-M manganese acetate; 0.02 A2° poly rA · oligo dT20; 2 × 10^{-1}-M [3H]-TP (5000 cpm/pmol); and 0.5% (v/v) Triton X-100. DNA synthesis was measured as previously described. Under these conditions incorporation of more than 0.2 pmoles/ml was considered positive in this assay. The virus-specific nature of the enzyme activity was confirmed by inhibition with antibody to mouse type-C virus reverse transcriptase.

Long-Term Bone Marrow Cultures

A summary of the optimal culture conditions is presented in Table 1. The contents of both a single adult mouse femur and tibia were flushed via a 22-gauge needle into a 1.0-mI Corning plastic flask in a total of 10.0 ml of Fisher's medium (GIBCO) supplemented with 25% horse serum (Flow Laboratories, Rockville, Md.) and 10^{-7}-M hydrocortisone sodium hemisuccinate (Solu-Cortef) (Upjohn, Kalamazoo, Mich.). Hydrocortisone reconstitution of horse serum stimulates and maintains both hemopoiesis and marrow preadipocyte differentiation in vitro. Cultures were incubated at 33° C in 7% CO2; the medium was changed, and the cultures were depopulated by weekly removal of 5.0 ml of medium containing nonadherent cells and replacement with 5.0 ml of fresh medium. Cultures were recharged at 28 days by addition of 1.0 × 10^7 fresh femur marrow cells according to published methods. Virus was added at this time for all studies reported here. Although cultures were initiated in 25% horse serum (Flow Laboratories) with 10^{-7}-M hydrocortisone sodium hemisuccinate, all were switched to 25% fetal calf serum with hydrocortisone at wk 5. Hemopoiesis in the absence of significant lipogenesis in corticosteroid-treated fetal calf serum has been reported. The calf serum switch increased virus titer and prolonged adherence of established hemopoietic cell islands.

Virus infection of marrow cultures was performed by addition of 10^5 PIU of each virus in 1.0 ml DME medium supplemented with 10% fetal calf serum and polybrene at 2.0 µg/ml.

Hemopoietic Cell Colony-Forming Assays

Weekly removed cells from marrow cultures were assayed for the number of granulocyte-macrophage progenitor cells (CFUc) by transfer of 10^4 or 5.0 × 10^4 cells to 1.0-mI cultures containing Alpha medium and 0.8% methyl cellulose (Dow Chemical). Cultures were incubated at 37° C, and 10-49 cell-containing clusters and colonies containing more than 50 cells were scored at 7 days under a Unitron inverted microscope. Individual colonies were removed from methyl cellulose cultures at 7 days for hematologic staining or were transferred for growth in suspension culture in Alpha medium supplemented with 10% WEHI-3 CSF. Colonies were prepared for microscopic examination by smearing onto glass coverslips and staining for myeloperoxidase, esterase-M (ASD-chloroacetate

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Table 1. In Vitro Conditions for Viral Induction of Continuous Acute Promyelocytic Leukemia Cell Lines From Mouse Bone Marrow

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimum for Current Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell number</td>
<td>2.0 × 10^7 inoculated cells (adult NIH/Swiss mice)</td>
</tr>
<tr>
<td>Site of cells</td>
<td>Femur and tibial marrow</td>
</tr>
<tr>
<td>Method of harvest</td>
<td>Flush marrow with 22-gauge needle; clumps not broken up</td>
</tr>
<tr>
<td>Culture flask</td>
<td>Corning 10.0-ml plastic</td>
</tr>
<tr>
<td>Medium</td>
<td>Fisher's medium, 10^7 X reconstituted (GIBCO), 25% horse serum (Flow Laboratories), any lot, 10^{-7}-M hydrocortisone sodium hemisuccinate (Solu-Cortef) added freshly each week. At wk 6 (42 days) 25% fetal calf serum (Flow Laboratories) substituted for horse. Hydrocortisone concentration freshly replenished.</td>
</tr>
<tr>
<td>Temperature</td>
<td>33° C, 7% CO2</td>
</tr>
<tr>
<td>Method</td>
<td>Cultures undisturbed for 7 days; 5.0-mI volume removed and fresh medium replaced weekly, virus added in 1.0-mI volume at wk 4 with fresh 10^7 marrow cells; weekly harvests transferred to suspension cultures in 10.0-mI plastic dishes</td>
</tr>
<tr>
<td>Suspension culture</td>
<td>Nonadherent cells centrifuged to pellet (1000 g for 10 min), transferred to Alpha medium (GIBCO) + 10% fetal calf serum (Colorado) + 10% WEHI-3 CSF at 37° C</td>
</tr>
</tbody>
</table>
substrate-specific), benzidine stain for hemoglobin, immunofluorescence for lysozyme, or Wright/Giemsa.32 Other CFUc cultures were grown in the absence of added CSF.

Pluripotent hemopoietic stem cells were assayed by spleen colony assay (CFUs) by inoculation of $10^3$ viable cells (by trypan blue dye exclusion) intravenously by tail vein into 1000-rad (cesium 137 source 663 kV, 140 rad/min) NIH/Swiss mice (30–33 g). Spleens were removed and colonies counted at 9–10 days after inoculation according to published procedures.33

Establishment of Continuous Virus-Induced Leukemic Cell Lines

Nonadherent cells removed weekly from long-term marrow cultures were centrifuged to a cell pellet (1000g for 5 min), and $10^9$–$10^7$ cells were transferred to liquid suspension culture in 10.0-mi plastic petri dishes (Falcon) in Alpha medium containing 10% WEHI-3 CSF and 10% fetal calf serum (Colorado). Cultures were fed weekly by removal of one-half the medium and replacement with an equal volume of fresh medium. Cells in the removed fraction were assayed weekly for total and differential cell counts and colony formation in methyl cellulose in the absence of CSF.

Tumorigenicity Studies

Cells were inoculated intraperitoneally to 1-day-old newborn NIH/Swiss mice or intravenously by tail vein to 30–33-g adult NIH/Swiss mice. Recipients were examined weekly for peripheral blood differential cell count and signs of anemia, splenomegaly, and weight loss. Postmortem examination was carried out on all animals and included Wright/Giemsa stain of spleen, liver, lymph node, and bone marrow touch preparations. Permanent pathologic sections were prepared from spleen, liver, bone marrow, and central nervous system according to published procedures.34

RESULTS

The effects of Friend, Abelson, BALB:virus-1 and Rauscher murine leukemia viruses on the growth and maintenance of hemopoiesis in vitro were first tested. Ten marrow cultures were infected with each virus at the time of fresh marrow recharging at wk 4, and 10 cultures were left uninfected. As shown in Fig. 1A, the total number of cells removed from recharged cultures infected at wk 4 with FLV-A or A-MuLV(M-MuLV) gradually increased above the numbers detected in control cultures. At 3 wk after virus infection (wk 7) there were no detectable differences in numbers or sizes of adipocyte colonies in the adherent microenvironment in virus-infected and control cultures; however, clear increases were observed in both total and relative numbers of immature granulocytic cells (myeloblasts, promyelocytes, and myelocytes) in FLV-A- or A-MuLV(M-MuLV)-infected cultures (Fig. 1A-B, Fig. 2A-B). There were also increases in the numbers of CSF-dependent colony-forming cells (CFUc) per $10^5$ cells removed in virus-infected cultures as compared with control cultures (Tables 2 and 3). However, comparable increases in CFUs in virus-infected cultures were not detected (Tables 2 and 3). These results confirm and extend those reported following infection of mouse marrow cultures with FLV-P.18

Generation of Acute Promyelocytic Leukemia Cell Lines by FLV-A or A-MuLV(M-MuLV) In Vitro

Weekly removed cell populations from virus-infected cultures or uninfected control cultures were transferred to Alpha medium supplemented with 10% fetal calf serum and 10% WEHI-3 CSF. As shown in Fig. 1A, the numbers of cells removed from FLV-A- or A-MuLV(M-MuLV)-infected cultures remained elevated between week 5 and 14, reaching a maximum at wk 7. Four of 10 marrow cultures infected with FLV-A and 4 of 8 cultures infected with A-MuLV(M-
MuLV) generated nonadherent cells between wk 6 and 7 that replicated at a rapid rate following transfer to suspension culture. Two A-MuLV(M-MuLV) cultures in this experiment were lost to contamination. The 7 wk harvest time correlated with detection of the greatest relative number of immature cells in that weekly removed fraction (Figs. 1B, 3A, and 3C; Tables 2 and 3). In contrast, nonadherent cells removed at wk 10 or 11 and transferred to identical conditions of continuous suspension culture did not establish.

Nonadherent cell populations removed from each of 10 Rauscher-virus-infected, 10 BALB: virus-1-infected, or 10 uninfected control cultures at wk 7 or 8 proliferated in suspension for 2–6 wk, whereas cells from these same cultures taken at 13 wk did not proliferate. However, none established as cell lines, and all differentiated to polymorphonuclear leukocytes and macrophages (Fig. 3E).

Cultures infected with Rauscher-MuLV demonstrated a pattern of morphologic alteration that was distinct from the patterns of both control cultures and cultures infected with FLV-A or A-MuLV(M-MuLV). Whereas nonadherent cell populations removed 3 wk after R-MuLV infection (wk 7) demonstrated less than 10% immature granulocytic forms, as compared with 50%–60% in FLV-A- or A-MuLV(M-MuLV)-infected cultures (Fig. 1B), these cells remained immature in suspension culture for longer periods than cells from uninfected control cultures. The relative numbers of immature cells harvested from R-MuLV cultures...
Fig. 2. A: Morphologic appearance of adherent cell population in a 7-wk bone marrow culture infected at wk 4 with FLV-A. B: Uninfected control. Fetal calf serum was substituted for horse serum at wk 5, as described in Table 1 (×200).
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Results are for a single flask generating the A(M) Ill cell line and are presented as described in the footnotes to Table 2.

Table 2. Culture Conditions During Generation of FLV-A 16-IV Cell Line In Vitro

<table>
<thead>
<tr>
<th>Days After Virus Addition</th>
<th>Adherent Cells</th>
<th>Surface Colonies†</th>
<th>Nonadherent Cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no. per flask/</td>
<td>Covered by## (no. per colony)</td>
<td>No. (X 10⁶)</td>
</tr>
<tr>
<td></td>
<td>no. per colony)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt;100/&gt;100</td>
<td>70</td>
<td>20.0</td>
</tr>
<tr>
<td>14</td>
<td>&gt;100/&gt;100</td>
<td>80</td>
<td>103.0</td>
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<tr>
<td>21</td>
<td>&gt;100/&gt;100</td>
<td>85</td>
<td>121.0*</td>
</tr>
<tr>
<td>28</td>
<td>76/&gt;100</td>
<td>60</td>
<td>67.0</td>
</tr>
<tr>
<td>45</td>
<td>53/&gt;100</td>
<td>15</td>
<td>17.0</td>
</tr>
<tr>
<td>63</td>
<td>45/85</td>
<td>&lt;5</td>
<td>5.6</td>
</tr>
<tr>
<td>105</td>
<td>6/18</td>
<td>&lt;5</td>
<td>7.5</td>
</tr>
</tbody>
</table>

*Cultures of NIH/Swiss adult marrow, 2.0 X 10⁷ cells per flask, were established at wk 0, and medium was changed as described in Table 1. At day 28, 1.0 X 10⁷ fresh tibia and femur marrow cells were added with 10⁸ PIU of FLV-A in a 1.0-ml volume. After incubation at 33°C, 7% CO₂ in this 1.0-ml shallow monolayer, 9.0 ml fresh medium was added. After 7 days the first determination was made (35 days after culture initiation). Results from a single flask generating cell line FLV-A 16-IV are presented here. Asterisk denotes cell harvest establishing the line.

†Adipocyte colonies per flask were scored by direct examination according to published procedures. Cell number per colony was taken as the mean of at least 20 colonies scored. The total number of colonies, if >50/culture, indicated adequate corticosteroid supplement for hemopoiesis in horse serum.25

‡At day 14 (42 days after initiation of cultures) 25% fetal calf serum was substituted for horse serum (Table 1). Sheets of round clusters of hemopoietic cells were observed to proliferate rapidly on the adherent cell monolayer. Results are an estimate of surface area covered.

§Total cells in weekly 5.0-ml removed volumes were counted and the percentage immature cells (‰), myeloblasts, promyelocytes, and myelocytes were scored by differential cell count on Wright/Giemsa-stained smears. The numbers of CFUc and CFUs were calculated as described in the Methods section. CFUs are the mean number of 9-day spleen colonies from 5 mice at each week, each receiving 1.0 X 10⁴ viable cells intravenously after 1000 rads. The upper limit of cell-proliferation in 10 uninfected control cultures was 60.0 X 10⁴ cells, 400 CFUs/10⁶ cells during the time shown.

increased between wk 9 and 15 (Fig. 1B) but decreased after that time. The morphology of granulocytic cells harvested during wk 9–15 was dysplastic, with large numbers of giant metamyelocytes, band forms with nucleoli, and basophilic promyelocytes not seen in control cultures. This distinctive myeloid dysplasia was also observed in BALB/virus-1-infected cultures with similar time courses.30

Table 3. Condition of Culture During Generation of A(M) Ill Cell Line in Vitro

<table>
<thead>
<tr>
<th>Days After Virus Addition</th>
<th>Adherent Cells</th>
<th>Surface Colonies†</th>
<th>Nonadherent Cells§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no. per flask/</td>
<td>Covered by## (no. per colony)</td>
<td>No. (X 10⁶)</td>
</tr>
<tr>
<td></td>
<td>no. per colony)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt;100/&gt;100</td>
<td>80</td>
<td>31.0</td>
</tr>
<tr>
<td>15</td>
<td>&gt;100/&gt;100</td>
<td>75</td>
<td>67.0</td>
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<tr>
<td>21</td>
<td>&gt;100/&gt;100</td>
<td>85</td>
<td>113.0*</td>
</tr>
<tr>
<td>28</td>
<td>51/&gt;100</td>
<td>40</td>
<td>110.0</td>
</tr>
<tr>
<td>43</td>
<td>27/&gt;100</td>
<td>35</td>
<td>273.0</td>
</tr>
<tr>
<td>81</td>
<td>16/&gt;100</td>
<td>30</td>
<td>176.0</td>
</tr>
<tr>
<td>110</td>
<td>7/47</td>
<td>10</td>
<td>63.1</td>
</tr>
</tbody>
</table>

*Results are for a single flask generating the A(M) Ill cell line and are presented as described in the footnotes to Table 2.
Fig. 3. Morphology of Wright/Giemsa-stained cells from the following: A: 7-wk culture of FLV-A-infected marrow culture freshly removed (×1000); B: same cell population as in A after 6 mo of continuous growth in suspension culture (×1000); C: 7-wk A-MuLV(M-MuLV)-infected culture cells freshly removed (×1000); D: same cell population as in C after 5 mo of continuous growth in suspension culture (×1000); E: 7-wk control uninfected marrow culture, cells freshly removed (×300).

Morphology, Histochemistry, and Biologic Properties of In-Vitro-Induced Granulocytic Leukemia Lines

As shown in Table 4, the morphology of a representative FLV-A- and A-MuLV(M-MuLV)-induced cell line was consistent with that of promyelocytic leukemia. There was a predominance of promyelocytes in both FLV-A 16-IV and A(M) III cell lines, with low numbers of mature PMLs and band forms continually seen. As shown in Fig. 3B, a subclone derived from FLV-A-induced cell line FLV-A 16-IV, designated FLV-A 16-IV clone 20, demonstrated 76% promyelocytes and 10% morphologically mature cells. The promyelocytes were morphologically atypical, with greater than 4 nucleoli per cell in 25%, atypical mitoses in 8%, multinucleated giant cell morphology in 8% and large cytoplasmic vacuoles in 63%.

Nonadherent cell populations removed from several FLV-A-infected primary marrow cultures as early as wk 5 (1 wk after virus inoculation) revealed these atypical promyelocytes. In retrospect, these cells were identical with the promyelocytes, constituting 70%-90% of cells in the established lines. FLV-A 16-IV demonstrated histochemically detectable lysozyme, esterase-M, and myeloperoxidase (Table 4). There was no detectable superoxide generating capacity (SGC) following stimulation with phorbol myristate acetate, whereas nonadherent cells from uninfected cultures as well as FLV-A-infected primary marrow cultures demonstrated SGC out to 20 wk.

Cell lines generated in vitro by A-MuLV(M-MuLV)-infected cultures showed a slightly different morphology. As shown in Fig. 3D, there were significantly more...
basophilic promyelocytes detected in cells of the A(M) III cell line, as compared with FLV-A 16-IV. In neither FLV-A- nor A-MuLV(M-MuLV)-induced cell lines were erythroblast, lymphoblast, or megakaryocytic morphologies detected.

The growth and colony-forming abilities of virus-induced cell lines were next evaluated. As shown in Table 4, both FLV-A 16-IV and A(M) III formed CFUc in the presence of WEHI-3 CSF with 50%–90% efficiency; however, both lines also formed colonies in the absence of CSF with 15%–63% efficiency. Colonies forming in CSF or forming independently did not terminally differentiate if removed from methyl cellulose, but they established as subclonal lines in medium supplemented with WEHI-3 CSF at 10%–15% efficiency.

**Virus Production in Primary Bone Marrow Cultures and Derived Suspension Lines**

To determine if the establishment of continuous cell lines from FLV-A- and A-MuLV(M-MuLV)-infected cultures at wk 7 was related to virus replication, tissue culture fluids from long-term primary marrow cultures were tested for reverse transcriptase (RT) activity. Nonadherent cell numbers in the FLV-A- and A-MuLV(M-MuLV)-infected cultures were 10–20-fold higher than the numbers detected in R-MuLV, BALB:virus-1 or control cultures at wk 7 (Fig. 1A). The medium from these cultures was tested as described in the Methods section, and 1.0-ml volumes were concentrated 100-fold by ultracentrifugation and tested for viral RT activity in the pellet virion fraction. As shown in Fig. 4, a peak of virus replication as reflected in RT activity was detected at wk 6–8. This time correlated with the greatest cell numbers in nonadherent fractions removed weekly (Fig. 1A).

**Table 4. Morphologic, Physiologic, and Histochemical Properties of FLV-A'16-IV and A(M) III In-Vitro-Induced Acute Promyelocytic Leukemia Cell Lines**

<table>
<thead>
<tr>
<th>Morphology (%)</th>
<th>Histochemistry (%)</th>
<th>Colony Formation in Agar</th>
<th>In Vivo Leukemogenicity</th>
<th>LD50/60</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLV-A 16-IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent line</td>
<td>6 81 6 2 3 2</td>
<td>2 &gt;90 2 0</td>
<td>76 18 10³</td>
<td></td>
</tr>
<tr>
<td>Subclone 3</td>
<td>2 91 0 3 3 1</td>
<td>0 &gt;90 1 0</td>
<td>81 25 N.T.</td>
<td></td>
</tr>
<tr>
<td>Subclone 12</td>
<td>1 83 5 2 5 4</td>
<td>1 &gt;90 3 0</td>
<td>93 20 N.T.</td>
<td></td>
</tr>
<tr>
<td>Subclone 20</td>
<td>8 76 0 1 0 3</td>
<td>1 &gt;90 1 0</td>
<td>71 17 10³</td>
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</tr>
<tr>
<td>Subclone 23</td>
<td>6 88 0 1 3 2</td>
<td>0 &gt;90 1 0</td>
<td>53 21 N.T.</td>
<td></td>
</tr>
<tr>
<td>A(M) III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent line</td>
<td>10 76 3 1 1 0</td>
<td>6 &gt;90 3 0</td>
<td>81 15 5.0 x 10³</td>
<td></td>
</tr>
<tr>
<td>Subclone 15</td>
<td>18 81 1 0 0 0</td>
<td>5 &gt;90 5 0</td>
<td>85 25 N.T.</td>
<td></td>
</tr>
<tr>
<td>Subclone 18</td>
<td>9 84 4 1 1 1</td>
<td>1 &gt;90 1 0</td>
<td>91 31 N.T.</td>
<td></td>
</tr>
<tr>
<td>Subclone 21</td>
<td>11 69 2 10 4 4</td>
<td>6 &gt;90 1 0</td>
<td>81 83 5.0 x 10³</td>
<td></td>
</tr>
</tbody>
</table>

* Coverslip smears stained with Wright/Giemsa were scored for percentages of cells with myeloblasts (Bl), promyelocytes (Pro), myelocytes (Myel), metamyelocytes (Meta), band forms (Bnd), and polymorphonuclear leukocytes and doughnut morphology (PMI). Results are mean percentages of 1000 cells scored on triplicate smears.

† Results are mean percentages of 1000 cells scored on triplicate coverslip smears tested by immunofluorescence for lysozyme (Lz) or histochemically for esterase-M (Est-M), myeloperoxidase (MPO), and leucocyte alkaline phosphatase (LAP) according to published procedures.26

‡ Triplicate cultures of 10², 10³, or 10⁴ cells in 1.0 ml of 0.8% methyl cellulose containing Alpha medium were scored for total number of >50-cell colonies at day 7 either in the presence or absence of 10% WEHI-3 CSF. Results are mean percentages of cells forming colonies. Both lines grew from 10³ cells/ml to 10⁴ cells/ml within 3 days, doubling time of 12–15 hr, saturation density of 1-1.2 X 10⁷ cells/ml.

§Serial 10-fold dilutions of cells in 0.1 ml of serum-free medium were inoculated into adult NIH/Swiss mice intravenously by tail vein. Results are doses of cells required to kill 50% of mice by 60 days (LD50/60). All dying mice had preceding rises in peripheral blood myeloblasts and died of acute granulocytic leukemia.

1 Uninfected NIH/Swiss marrow cultures were harvested weekly as described in the Methods section. Results are for nonadherent cells harvested at wk 7 (3 wk after rechallenge) in primary culture and an additional 10 days culture in suspension in Alpha medium supplemented with 10% WEHI-3 CSF (total 8.5 wk).
Fig. 4. Virus replication in FLV-A-, A-MuLV(M-MuLV)- and R-MuLV-infected marrow cultures. Results are means of at least three cultures tested at each week for viral RT activity following infection at wk 4 with marrow recharge for the following: closed circles, FLV-A-infected; squares, A-MuLV(M-MuLV)-infected; triangles, R-MuLV-infected; open circles, uninfected control cultures. Results are presented as pmoles/[^H]TP incorporated per milliliter of culture fluid removed at the time and are not corrected for total cell number in the culture.

There was no detectable difference in virus production between those cultures (4 of 10 FLV-A-infected and 4 of 8 A-MuLV(M-MuLV)-infected) that generated a continuous leukemia cell line and those that did not. The lower RT levels for R-MuLV-infected cultures (Fig. 4) may reflect both decreased replication of virus in those cultures and the 10–20-fold lower total cell numbers in those cultures. However, in previous studies from this laboratory in which higher cell numbers were generated by infection of other NIH/Swiss marrow cultures with the same stocks of R-MuLV or BALB: virus-I and in which RT activity in those cultures was also comparably higher, no continuous cell lines were generated, and only the dysplastic granulocytic changes were seen.

Host Range of Cell-Line-Released Viruses

Supernatants from continuous cell lines FLV-A 16-IV and A(M) III after 60 days growth of each line in suspension culture were filtered free of cells, and serial 10-fold dilutions were titered into polybrene-treated cultures of NIH/3T3, BALB/3T3, or NRK cells. After 7 days these fibroblast cultures had their medium changed, and 24-hr supernatants were assayed for RT activity. In parallel assays the same cell line supernatants were transferred to polybrene-treated cultures of K-NIH, K-BALB, or K-NRK cells, and 7-day fresh 24-hr supernatants were titered for focus formation by rescued KiMSV on BALB/3T3, NIH/3T3, or NRK according to published procedures.

The results demonstrated growth of the virus released by FLV-A 16-IV on all three cell lines, with preferential N-tropism (3 log dilutions RT positivity on NIH/3T3, 1 log dilution on NRK or BALB/3T3; 4 log dilutions positivity of KiMSV focus formation by virus rescued from K-NIH, 2 log dilutions from K-BALB, 1 log dilution from K-NRK). In contrast, the virus released by A(M) III demonstrated a slight B-tropism (2 log dilutions RT positivity on BALB/3T3, 1 log dilution on NIH/3T3, none detected on NRK; 3 log dilutions positivity of KiMSV focus formation by virus rescued from K-BALB, 1 log dilution from K-NIH, none from K-NRK). Further studies with cell-line-released viruses will be reported separately.
Pathogenicity of In Vitro Virus-Induced Continuous Promyelocytic Cell Lines

FLV-A- and A-MuLV(M-MuLV)-induced continuous tissue culture lines were tested for leukemogenicity in both newborn and adult syngeneic mice. As shown in Table 5, 50% of newborn mice inoculated with either FLV-A 16-IV or A(M) III developed fatal acute myelogenous leukemia by 23 days following inoculation of 10^6 cells. In contrast, newborn mice receiving cell-free filtrates of the viruses released by FLV-A 16-IV and A(M) III cells grown for over 100 days in suspension developed characteristic Friend erythroleukemia or Abelson virus lymphoma, respectively, at significantly later times.

To confirm the pathogenicity of the suspension culture cells, adult NIH/Swiss mice were inoculated by tail vein with 10^3, 10^4, 10^5, or 10^6 cells of each cell line. As shown in Table 5, mice receiving 10^6 FLV-A 16-IV or A(M) III cells developed splenomegaly and increased numbers of peripheral blood peroxidase-positive myeloblasts and promyelocytes within 6 wk. However, 30% of mice survived past 9 wk, with clearing of peripheral blood blast cells. Spleen touch preparation examination of peripheral blood, bone marrow, liver, and central nervous system of dying newborn and adult mice revealed infiltration of organs with promyelocytes and myeloblasts. Spleen cells removed from a representative adult mouse dying of disease induced by FLV-A 16-IV were transferred to other adult and newborn mice in serial 10-fold dilutions. A similar disease was induced in 100% of newborn mice but in only 50% of adult mice. Mice receiving lower numbers of tissue-culture-grown cells or passaged cells from dying mice developed leukemia at lower frequency. With FLV-A 16-IV or A(M) III the morphology of disease in vivo was indistinguishable from that of the in vitro tissue culture line.

At the present time it is impossible to be certain of the donor cell origin of the granulocytic leukemias induced in both adult and newborn mice receiving FLV-A 16-IV or A(M) III cells. Karyotyping studies in progress with male NIH/Swiss

### Table 5. Characteristics of Diseases Induced Following Inoculation of FLV-A 16-IV or A(M) III Cells or Cell-Line-Released Virus

<table>
<thead>
<tr>
<th>Inoculum (age)</th>
<th>Adult Recipients</th>
<th>Newborn Recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days to Death (median)</td>
<td>Spleen Weight (g)</td>
</tr>
<tr>
<td>Cell line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FLV-A 16-IV (112 days)</td>
<td>63</td>
<td>0.25-0.39</td>
</tr>
<tr>
<td>A-MuLV(117 days)</td>
<td>41</td>
<td>0.15-0.27</td>
</tr>
<tr>
<td>Cell-line-released virus</td>
<td>&gt;200</td>
<td>0.16-0.18</td>
</tr>
<tr>
<td>FLV-A 16-IV (180 days)</td>
<td>&gt;200</td>
<td>0.17-0.19</td>
</tr>
<tr>
<td>A-MuLV(M-MuLV) (117 days)</td>
<td>&gt;200</td>
<td>0.16-0.18</td>
</tr>
<tr>
<td>Stock inoculum virus</td>
<td>&gt;200</td>
<td>0.16-0.18</td>
</tr>
<tr>
<td>Medium control</td>
<td>&gt;200</td>
<td>0.16-0.18</td>
</tr>
</tbody>
</table>

*Results are for inoculation of 10^6 cells intravenously by tail vein to adult mice or intraperitoneally on the first day of life to newborn mice. Median time to death is the day of death of 50% of a group of at least 10 adults or a litter of 10 newborn mice. Spleens, marrow, and lymph nodes were examined at death or sacrifice (for controls), according to published procedures. **N** = normal, + = enlarged. **%M** = percentage of marrow differential cell count consisting of granulocytic cells. Age represents days in culture of cell line or filtered supernatant at time of inoculation for tumorigenicity assay.

†Results are for inoculation of fresh cell-free filtrate from cell lines growing in suspension culture for times indicated with passage of cells every 4 or 5 days. Virus stocks used to generate cell lines in vitro are as described in the Methods section. The parent stocks FLV-A and A-MuLV(M-MuLV) were standardized to 10^7 PIU/ml, while viruses released by cell lines were 10^7 PIU/ml. Each adult or NIH/NH/Swiss mouse received 0.1 ml total volume.
mouse Dexter-culture-generated lines inoculated into female recipients should resolve the question. However, the clear difference in phenotypes of leukemias resulting from inoculation of cell lines and phenotypes of leukemias resulting from inoculation of viruses released by those cell lines strongly suggests that FLV-A 16-IV and A(M) III cells produce granulocytic leukemia in vivo.

DISCUSSION

This report details the first in vitro induction of continuous cell lines of acute myelogenous leukemia. In a previous study, Dexter et al. demonstrated generation of large numbers of immature granulocytic cells in bone marrow cultures infected with FLV-P. However, transfer of these cells to adult or newborn mice induced granulocytic leukemia in only some cases, and the disease that predominated in subsequent generations was erythroleukemia induced by the virus. Thus, the in-vitro-generated granulocytic leukemia cells in their study could neither be continually passaged in vivo nor be established in culture. Differences between our system and the system reported by Dexter include our addition of corticosteroid and a switch to fetal calf serum after wk 5. Fetal calf serum increases virus proliferation in vitro as compared with horse serum. In more recent studies we have obtained 100% frequency (16/16) of promyelocyte cell lines using FLV-A in NIH/Swiss marrow cultures. Thus, 20 FLV-A in-vitro-induced promyelocyte lines have been established.

In the present studies, malignant phenotypically granulocytic cell lines were induced in vitro in a microenvironment that greatly favored granulocytic differentiation by each of two viruses that normally cause nongranulocytic leukemias in vivo, FLV-A and A-MuLV (M-MuLV). The disease produced following transfer of these promyelocytic cell lines to adult or newborn mice was phenotypically granulocytic; however, viruses released by the cell lines did not induce granulocytic leukemia, but produced diseases indistinguishable from those caused by inoculation of each of the parent viruses. Thus, long-term growth of FLV-A and A-MuLV (M-MuLV) in promyelocytes (7 wk in primary culture and an additional 14–17 wk in suspension culture) did not detectably alter the in vivo pathogenicity or phenotypic preference of differentiation of the resultant virus-induced disease. These data provide evidence that in vivo production of erythroleukemogenesis by FLV-A and lymphoid leukemia by A-MuLV (M-MuLV) is attributable to prevailing selection pressures in the hematopoietic microenvironment rather than to inhibition of virus replication and/or transformation in granulocytic cells. Granulocyte-macrophage phenotypic diseases have been reported as rare in vivo events with these viruses.

Adult mice receiving serially passaged or lower numbers of FLV-A 16-IV cells developed granulocytic leukemia at lower frequency. Furthermore, some of lethally irradiated adult mice receiving 10⁶ FLV-A 16-IV cells intravenously did not die of irradiation-marrow death at 14 days (as did controls) or of granulocytic leukemia. These “reconstituted” mice survived in excess of 60 days, and many then died of FLV-A erythroleukemia. These results are similar to those of Dexter et al. and suggest that granulocytic leukemia cells induced in vitro by FLV-A may retain the ability to respond to strong differentiation stimuli in vivo. No evidence for erythroid or lymphoid differentiation of FLV-A 16-IV cells was detected following in vitro
stimulation with erythropoietin or bacterial lipopolysaccharide and sheep red blood cells. Further studies on the differentiation responses of these cells are in progress.

The present marrow culture system is known to sustain proliferation of pluripotent stem cells that form erythroid as well as myeloid spleen colonies; however, cells differentiating in vitro shift to the granulocyte series. This shift has been attributed to the presence of large numbers of macrophages, endothelial cells, and fibroblasts that produce myeloid stimulatory molecules. When viruses that cause primarily nongranulocytic malignancies in vivo or in fresh marrow immediately following explant are introduced into a microenvironment in vitro that selects for a disproportionate infection of proliferating committed granulocytic target cells, granulocytic leukemia develops. This might simply be a result of transformation of committed myeloid cells. Alternatively, pluripotent hemopoietic stem cells in the cultures may be the critical target cells with granulocyte differentiation during transformation. Evidence in favor of the latter mechanism is as follows: (1) cell lines were established from early harvested nonadherent cells (wk 7), a time when CFUs are abundant, rather than from late harvests (wk 12), when CFUs are less prevalent; (2) infection of isopycnotic-gradient-purified fractions of fresh marrow or wk 7 Dexter-culture marrow, enriched for CFUc, and transferred to viscous medium with CSF, produced more than 90% productively infected granulocytic colonies, but a very low frequency of cell lines generated (manuscript in preparation); (3) infection of Dexter cultures at later time periods (wk 12 or 15) when CFUs were depleted resulted in generation of no leukemia lines (manuscript in preparation).

Rauscher-MuLV-infected cultures in the present experiments generated lower titers of virus, but they have been shown to contain an equally high percentage of infected cells, as measured by infectious center assay. Thus, a higher level of individual cell virus replication, rather than numbers of cells infected or genetic factors specific to FLV-A or A-MuLV(M-MuLV), may determine transformation. If replication is the critical variable, then variant viruses with high replication propensity or improved in vitro culture conditions with longer exposure of target cells to poorly replicating viruses may lead to stably transformed cell lines. Alternatively, if virus-specific genetic functions or cell populations absent from Dexter cultures are critical to R-MuLV or BALB: virus-1 leukemogenesis in NIH/Swiss marrow, then higher titers of these viruses, prior x-ray or chemical mutagen exposure of marrow in vivo, or larger numbers of cultures tested would not be expected to demonstrate generation of leukemic cell lines. These studies are in progress.

Another more critical variable for Dexter-system studies appears to be mouse strain genotype. Culture of marrow from some specific strains using the present steroid-reconstituted serum method led to spontaneous induction of replication of endogenous ecotropic virus and generation of continuous promyelocytic leukemia cell lines in a very high proportion of cultures (manuscript in preparation).

The morphologic and biologic alterations observed following RNA type-C virus infection of granulocytic cells in long-term marrow culture are similar to those seen in human myeloproliferative disorders. In particular, the characteristics of granulocytic cells infected with R-MuLV or BALB: virus-1 resembled preleukemic dysmyelopoiesis. Studies are in progress to determine how this stable preleukemic condition caused by one virus can be driven to overt leukemia by another.
ACKNOWLEDGMENT

We thank Drs. George P. Canellos, T. M. Dexter, Peter M. Ralph, and Malcolm A. S. Moore for helpful discussions.

ADDENDUM

After this manuscript had been submitted, we learned of recent reports from Drs. T. M. Dexter, N. G. Testa, and N. M. Teich of the establishment of a continuous in vitro granulocytic cell line from FLV-P-infected B6D2 F1 mouse marrow cultures and a continuous in vitro line of IgM-synthesizing pre-B cells from Abelson-(Moloney-MuLV)-infected BALB/c mouse marrow cultures.

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In vitro induction of continuous acute promyelocytic leukemia cell lines by Friend or Abelson murine leukemia virus

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