**In Vitro Growth of an Attenuated Mouse Leukemia Virus**

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**SUMMARY**

The replication of an attenuated and a live mouse leukemia virus was examined in short-term and long-term mouse cell cultures. The viruses were harvested from the culture fluids of chronically infected mouse cell lines or from the spleen of a mouse with virus-induced erythroblastosis. The attenuated Kirsten mouse leukemia virus replicated poorly in S\(^{L^-}\) cells and rescued significantly lower titers of Moloney sarcoma virus from S\(^{L^-}\) cells than did Rauscher mouse leukemia virus or live Kirsten mouse leukemia virus. A decrease in the number of virus-releasing cells, autointerference between attenuated and live viruses, or a requirement for dual infection of target cells did not account for the replication defect of the attenuated virus.

**INTRODUCTION**

Attenuated MuLV\(^4\) have been isolated from several virus-producing mouse fibroblast cell lines (2, 14, 16, 24, 25, 31—33). We have recovered an attenuated MuLV, KiMuLV\(^-\), from the culture fluids of a mouse line that has been used as a virus source for almost 6 years (14). KiMuLV\(^-\) failed to cause erythroblastosis or lymphomas in mice or rats but effectively protected against the induction of these diseases by homologous or heterologous live virus.

The mechanisms by which the attenuated viruses prevent leukemia-lymphoma induction are unknown. The attenuated viruses may interfere with the replication of live virus in target cells (12) or may elicit antiviral antibodies which, in turn, prevent virus spread in the host and thereby progression of the disease (6). The attenuated viruses may also initiate infection in host cells under restrictive conditions resulting in the production of noninfectious progeny. Since any of these alternatives are difficult to test in vivo, we have studied the replication of an attenuated and the corresponding live MuLV in cell cultures. The results suggest a marked defect in the replication of KiMuLV\(^-\).

**MATERIALS AND METHODS**

**Cells and Virus.** The MECI line was derived in 1967 from C3Hf/Gs mouse embryo cell cultures infected with KiMuLV (14, 29). The line was maintained in cultures since then and released KiMuLV\(^-\) into the culture fluids (14). KiMuLV\(^-\) has the structure of mouse C-type viruses and a buoyant density of 1.15 g/ml in sucrose equilibrium gradients (Refs. 17, 19; unpublished data). A single stock of KiMuLV\(^+\) was obtained from a cell-free spleen extract of a C3Hf/Gs mouse with virus-induced erythroblastosis (13). RaMuLV was harvested from the culture fluids of the JLS-V9 line which was kindly provided by Dr. Frank Rauscher, Bethesda, Md. (22, 32). KiMuLV\(^-\) and RaMuLV were prepared from subconfluent MECI and JLS-V9 cultures, respectively. Culture fluids were harvested 24 hr after the last medium change and passed through a Nalgene suction-filtration unit (0.45-µm pore size). Aliquots of the filtrate were stored at \(-65^\circ\).

Cloned NRK cells were obtained from Dr. Kenneth Somers, Houston, Texas (7). Swiss mouse 3T3FL cells and the M-MSV-transformed S\(^{L^-}\) derivative of 3T3FL cells were gifts of Dr. Robert Bassin, Bethesda, Md. (3, 4). The RSV-transformed XC line of rat cells was a gift of Dr. S. Chandra, Chicago, Ill.

MECI cells were propagated in Medium 199 containing 10% fetal calf serum; penicillin, 100,000 units/liter, streptomycin, 100,000 µg/liter (Grand Island Biological Co., Grand Island, N. Y.) and polymyxin B, 125,000 units/liter (Pfizer Inc., New York, N. Y.). NRK, 3T3FL, JLS-V9, and XC cells were grown in Eagle's MEM with Earle's salts (Flow Laboratories, Rockville, Md.) and supplemented as described above. The medium for XC cells also contained 0.8 g/liter rather than the standard amount (2.2 g/liter) of sodium bicarbonate. The growth medium for S\(^{L^-}\) cells was modified McCoy's SA medium with 10% fetal calf serum, penicillin, and streptomycin as outlined above; amphotericin B, 1 mg/liter (Fungizone; E. R. Squibb and Sons, Princeton, N. J.); and garamycin, 5 mg/liter (Gentamycin; Schering Corp., Bloomfield, N. J.) also were added (3).

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\(^2\)Predoctoral trainee supported by NIH Training Grant HD-00297 from the National Institute of Child Health and Human Development.

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\(^4\)The abbreviations used are: MuLV, murine leukemia virus(es); KiMuLV, Kirsten MuLV; KiMuLV\(^-\), attenuated KiMuLV; MECI, C3H mouse cell line chronically infected with KiMuLV; KiMuLV\(^+\), live KiMuLV; RaMuLV, Rauscher mouse leukemia virus; M-MSV, Maloney murine sarcoma virus; MSV, murine sarcoma virus; MEM, minimal essential medium; PFU, plaque-forming units; FFU, focus-forming units.

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All cells were grown at 37° in 5% CO₂ atmosphere in 4-oz glass pharmacy bottles or 25-sq cm plastic flasks (Falcon Plastics, Oxnard, Calif.). Cells were harvested in a clinical centrifuge (1000 X g for 10 min) and resuspended in fresh growth medium by pipetting. Cell lines were harvested by washing with phosphate-buffered saline free of CA “ and Mg”, followed by the addition of 0.05% trypsin and 0.2% EDTA. The cell suspensions were passed through a 20-gauge needle to disperse clumps and were diluted with growth medium. When large numbers of cells were needed, 32-oz pharmacy bottles or roller bottles were used. Stocks of cells were tested biweekly for Mycoplasma contamination by the method of Hayflick (11). Samples of culture fluid and loose cells were incubated aerobically and anaerobically on PPLO agar and examined for colonies at X20 after 1 and 2 weeks. Only Mycoplasma-free cell cultures were used.

Infectivity Studies. Cells were seeded sparsely in an appropriate volume of medium. The cultures were treated the next day with DEAE-dextran as described for the MSV assay, washed, and inoculated with a minimal amount (to promote adsorption) of a MuLV stock previously titered by the S’L” plaque assay (see below). The multiplicity of infection was determined by dividing the PFU of added MuLV by the number of viable cells seeded.

Focus Assay for MSV. Plastic tissue culture dishes (50 mm, Falcon) were seeded with 2 X 10⁸ viable NRK cells in 4 ml MEM on Day 0 (9, 20, 27). The medium was replaced 1 day later with 4 ml fresh MEM containing 25 μg DEAE-dextran per ml (Pharmacia Laboratories Inc., Piscataway, N. J.) (27). The medium was left for 1 hr at 37°, removed, and the cultures were washed with MEM. Duplicate cultures received 0.5 ml of virus inoculum serially diluted in MEM; control cultures received MEM only. The dishes were shaken without lifting to distribute the inoculum evenly. They were incubated at 37° for 90 min with shaking every 15 min, and 3.5 ml MEM were added to each dish. Medium was changed on Day 3 or 4. The cultures were barely confluent on Day 6 or 7 when they were washed with 0.9% NaCl solution, fixed with 100% methanol, and stained with Giemsa. Foci of transformed cells appeared as dense clusters of darkly stained cells at X30. Approximately 200 foci could be accurately counted in a single dish.

S’L” Plaque Assay for MuLV. The procedure of Bassin et al. (3, 4) was followed. Twenty-five-sq cm flasks received 1.25 X 10⁸ viable S’L” cells in 3 ml McCoy’s Medium on Day 0. The cultures were infected as described for the MSV focus assays on Day 1, without DEAE-dextran treatment. After adsorption for 1 hr with periodic shaking, each flask was fed with 2.5 ml McCoy’s medium which was changed every 2 to 3 days. The cultures were fixed and stained on Day 7 or 8. Sites of MuLV infection appeared as discrete, sparsely populated areas containing small clusters of round, heavily stained cells. Up to about 150 plaques could be counted in a single flask.

XC Plaque Assay for MuLV. The method of Rowe et al. (23) was modified to determine the percentage cells in a culture that released MuLV capable of forming XC syncytia. The cells to be studied were seeded in 50-mm dishes at 100 cells/dish on Day 0. The dishes were examined with a Unitor inverted microscope on Day 3 or 4. Circles were marked around cell colonies from beneath. XC cells, 4 X 10⁸, in XC-MEM were added to each dish and incubation continued. The cultures were stained with Giemsa on Day 6 or 7, and each colony location was scored for associated syncytia.

RESULTS

Virus Release. To account for the loss of leukemogenicity by KiMuLV, we considered that virus production by MECI cells may have been reduced markedly during their 6-year passage in vitro (14). Rowe et al. (23) developed a plaque assay to measure MuLV using syncytia formation in Rous sarcoma virus-transformed XC-rat cell cultures (28). The test was modified for the present work to determine the proportion of virus-releasing cells in MECI and JLS-V9 cultures (see "Materials and Methods").

Dishes were seeded sparsely, allowed to develop into small colonies, and overlaid with XC cells on Day 3 or 4. A colony was scored as an infectious center, i.e., as a colony of virus-releasing cells, if one or more XC syncytia developed within a marked area. Fifty-six of 57 MECI colonies and 72 of 73 JLS-V9 colonies registered as infectious centers, whereas none of the 80 uninfected 3T3FL colonies were associated with XC syncytia. Assuming that low-density cell plating does not select virus-releasing cells, a reduction in the proportion of virus-releasing cells can be ruled out as an explanation for virus attenuation.

S’L” Assays. This rapid (7-day) method is based on the rescue of MSV and release of progeny MuLV from the transformed S’L” cells after superinfection with MuLV (3, 4, 21). Plaque counts (PFU/0.5 ml) following MuLV infection provide a direct measure of the input dose of infectious MuLV, and culture fluids can be assayed on fresh S’L” cell cultures to measure infectious output MuLV or on NRK cells to determine focus formation (FFU/0.5 ml) by rescued M-MSV. NRK cells were chosen for focus assays because these cells do not require dual infection with MSV and MuLV, and MSV titers can therefore be quantitated in the absence of excess MuLV (7, 18, 20).

When the yields of infectious progeny KiMuLV and RaMuLV were compared, the ratio of output PFU/input PFU was 16-fold greater for RaMuLV (0.33) than for KiMuLV (0.02) (Table 1, Experiment A). The ratio of FFU/input PFU was also far greater for RaMuLV (71.5) than for KiMuLV (0.03). It is unlikely that some or all output MuLV represented residual input virus after 6 days in culture at 37° and 2 medium changes, since MuLV are known to become inactivated under these culture conditions (17). The growth of KiMuLV and that of its homologous, live virus (KiMuLV’), was modified for the present work to determine the excess MuLV (7, 18, 20).

When the yields of infectious progeny KiMuLV and RaMuLV were compared, the ratio of output PFU/input PFU was 16-fold greater for RaMuLV (0.33) than for KiMuLV (0.02) (Table 1, Experiment A). The ratio of FFU/input PFU was also far greater for RaMuLV (71.5) than for KiMuLV (0.03). It is unlikely that some or all output MuLV represented residual input virus after 6 days in culture at 37° and 2 medium changes, since MuLV are known to become inactivated under these culture conditions (17). The growth of KiMuLV” and that of its homologous, live virus (KiMuLV”) were compared in Table 1, Experiment B. Again, the ratio of output FFU/input PFU was approximately 22-fold greater for KiMuLV” than for KiMuLV”. Focus formation by rescued M-MSV was not tested in Experiment B.

Prolonged in vitro propagation may reveal growth differences between an attenuated and live MuLV not detectable by short-term assays. For this purpose, duplicate 3T3FL cultures were infected at a multiplicity of infection of 1.6 X 10⁻⁴ with KiMuLV” as described. The cultures were designated 3T3− or 3T3+ cells, and maintained for 12 passages. Roller bottles
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Table 1

Replication of KiMuLV - , KiMuLV + , and RaMuLV in S+L - cells with concomitant M-MSV rescue

S+L - cell cultures (1.25 x 10^6 cells/flask) were infected with KiMuLV - (undiluted MECI culture fluids), KiMuLV + (10^-3 dilution of a spleen extract from a C3H/Gs mouse with erythroblastosis), RaMuLV (10^-3 dilution of JLS-V9 culture fluids), KiMuLV + (3T3) (undiluted 3T3/- culture fluids), and KiMuLV - (3T3) (10^-3 dilution of 3T3/+ culture fluids). Plaques were counted on Day 6 to determine input MuLV (PFU/0.5 ml). Pooled, filtered S+L - culture fluids were assayed on freshly seeded S+L - cells for infectious output MuLV (PFU/0.5 ml) and in fresh NRK culture fluids), and KiMuLV - (3T3) (10^-2 dilution of 3T3/+ culture fluids). Plaques were counted on Day 6 to determine input MuLV (PFU/0.5 ml). Pooled, filtered S+L - culture fluids were assayed on freshly seeded S+L - cells for infectious output MuLV (PFU/0.5 ml) and in fresh NRK cultures for rescued M-MSV (FFU/ml).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inoculum</th>
<th>Input MuLV (PFU/0.5 ml)</th>
<th>Output MuLV (PFU/0.5 ml)</th>
<th>Rescued M-MSV (FFU/0.5 ml)</th>
<th>Output MuLV^a/ input MuLV</th>
<th>Rescued M-MSV^b/ input MuLV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>KiMuLV - (10^6)</td>
<td>64, 59</td>
<td>2, 0</td>
<td>&gt;200, &gt;200^c</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>RaMuLV (10^-3)</td>
<td>124, 143</td>
<td>50, 39</td>
<td></td>
<td>0.33</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>McCoy's medium</td>
<td>0, 0</td>
<td>0, 0</td>
<td></td>
<td>0.07</td>
<td>NT</td>
</tr>
<tr>
<td>B</td>
<td>KiMuLV - (10^6)</td>
<td>28, 33</td>
<td>2, 2</td>
<td></td>
<td>NT^d</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>KiMuLV + (10^-2)</td>
<td>35, 29</td>
<td>55, 44</td>
<td></td>
<td>1.55</td>
<td>NT</td>
</tr>
<tr>
<td>C</td>
<td>KiMuLV + (3T3) (10^6)</td>
<td>18, 9</td>
<td>7, 9</td>
<td>127, 139</td>
<td>0.6</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>KiMuLV + (3T3) (10^-3)</td>
<td>20, 15</td>
<td>63, 64</td>
<td>176, 154</td>
<td>36</td>
<td>943</td>
</tr>
<tr>
<td></td>
<td>3T3FL fluids</td>
<td>0, 0</td>
<td>0, 0</td>
<td></td>
<td>0.07</td>
<td>NT</td>
</tr>
</tbody>
</table>

^a Sum of output PFU/sum of input PFU.

^b Sum of FFU/sum of input PFU.

^c Where foci were too numerous to count, NRK culture was assumed to contain more than 200 foci.

^d NT, not tested.

were then seeded with 2 x 10^7 3T3FL, 3T3/-, and 3T3/+ cells to obtain large virus pools. All bottles were 75% confluent 4 days later when 24-hr culture fluids were harvested, filtered, and frozen as virus stocks. The virus released by 3T3/- cells was designated KiMuLV - (3T3); that released by 3T3/+ cells was designated KiMuLV + (3T3). The 2 virus stocks were assayed in S+L - cells for plaque formation and M-MSV rescue. In preliminary experiments, S+L - cultures were infected with serial 10-fold dilutions of KiMuLV - (3T3) or KiMuLV + (3T3); control cultures received aliquots of filtered 3T3FL culture fluids only. Infection with undiluted KiMuLV - (3T3) resulted in a small number of input plaques, whereas the KiMuLV + (3T3) stock required a 1000-fold dilution to yield a comparable number of plaques. The ratio of output PFU/input PFU was 60-fold greater for the KiMuLV + (3T3) stock as compared to the KiMuLV - (3T3) stock (Table 1, Experiment C). Likewise, the ratio of rescued M-MSV/input PFU was 95-fold greater for the KiMuLV + (3T3) stock than for KiMuLV - . It should be noted that KiMuLV + and KiMuLV - exhibited significantly higher output/input ratios after passage through 3T3FL cells than after short-term growth in S+L - cultures. A comparison of these ratios reveals a 22-fold increase of the output PFU/input PFU for KiMuLV + (3T3) relative to KiMuLV - and a 9-fold increase for KiMuLV + (3T3) relative to KiMuLV - (Table 1, Experiments B and C). Moreover, the ratio of output PFU/input PFU for KiMuLV - (3T3) was 330-fold greater than that for KiMuLV - .

Interference and Complementation. The KiMuLV - population may consist largely of noninfectious particles which interfere with the replication of infectious virions. Bondurant et al. (5) have demonstrated such autointerference by noninfectious particles of M-MSV(MuLV) after serial high density passages. To test this possibility, S+L - cultures were infected with KiMuLV - , KiMuLV + , or mixtures of these. Duplicate S+L - cultures received 0.5-ml of the appropriate virus inoculum on Day 1, and were fed on Day 4. Culture fluids were pooled, filtered, and frozen on Day 7, when the cultures were stained and scored. Serial 10-fold dilutions of the fluids were assayed for plaque formation on fresh S+L - cell cultures. The yield of infectious virus after infection with KiMuLV + alone was not altered by coinfection with KiMuLV - at any of the doses tested (Table 2). If interfering particles were indeed present in significant titers, cultures coinfected with KiMuLV + and KiMuLV - should release lower titers of infectious progeny virus than do cultures infected with KiMuLV + alone. The data do not provide evidence for the presence of interfering particles in the KiMuLV - population.

Dose Response of Plaque Formation. The attenuated virus may be "defective" in the sense that 2 or more viroms must coinfect a cell for infectious progeny to result. S+L - plaque formation by KiMuLV - would then exhibit a multihit dose response, i.e., when cell cultures are infected with serial dilutions of a virus stock the apparent titer decreases as the dilution factor increases (10). To test this hypothesis, parallel S+L - cultures were infected with serial 2-fold dilutions of KiMuLV - or KiMuLV + . The data in Chart 1 are plotted according to the method of Hartley and Rowe (10). If plaque formation exhibits a single-hit response, a line drawn through the experimental points will have a slope of 0, whereas a 2-hit response will produce a line with a slope of -1. The regression lines in Chart 1 were fitted to the data points by the method of least squares, with all points weighted equally. The results indicate that plaque formation by KiMuLV - or KiMuLV + in S+L - cells requires only 1 hit.

DISCUSSION

The present experiments compare the in vitro growth of KiMuLV - with a highly leukemogenic, homologous virus (KiMuLV + ) and RaMuLV. KiMuLV + is a highly infectious
Table 2
Coinfection of S′L′ cultures with KiMuLV− and KiMuLV+

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Input MuLV (PFU/0.5 ml)</th>
<th>Output MuLVa (PFU/0.5 ml)</th>
<th>Corrected output MuLVb (PFU/0.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KiMuLV−</td>
<td>1:10</td>
<td>TNTC</td>
<td>72, 91</td>
</tr>
<tr>
<td>KiMuLV−</td>
<td>1:10</td>
<td>TNTC</td>
<td>89, 94</td>
</tr>
<tr>
<td>KiMuLV−</td>
<td>1:10</td>
<td>TNTC</td>
<td>57, 72</td>
</tr>
<tr>
<td>KiMuLV−</td>
<td>Undiluted</td>
<td>TNTC</td>
<td>86, 78</td>
</tr>
<tr>
<td>KiMuLV−</td>
<td>Undiluted</td>
<td>66, 16</td>
<td>23, 13</td>
</tr>
<tr>
<td>KiMuLV+</td>
<td>1:10</td>
<td>TNTC</td>
<td>72, 91</td>
</tr>
<tr>
<td>KiMuLV+</td>
<td>1:10</td>
<td>TNTC</td>
<td>89, 94</td>
</tr>
<tr>
<td>KiMuLV+</td>
<td>1:10</td>
<td>TNTC</td>
<td>57, 72</td>
</tr>
<tr>
<td>KiMuLV+</td>
<td>Undiluted</td>
<td>TNTC</td>
<td>86, 78</td>
</tr>
<tr>
<td>KiMuLV+</td>
<td>Undiluted</td>
<td>66, 16</td>
<td>23, 13</td>
</tr>
</tbody>
</table>

a All output PFU were tested at a dilution of 1:100 with the exception of the test with undiluted KiMuLV− alone (Line 5) which was assayed undiluted.
b Output PFU x dilution factor.
c TNTC, too numerous to count.

Growth of Attenuated Mouse Leukemia Virus

Lieberman et al. (16) have likewise demonstrated that all cells in a culture releasing attenuated radiation-leukemia virus behave as infectious centers. The short-term replication experiments using S′L′ plaque assays (3, 4) revealed that the relative number of infectious progeny virions was significantly reduced for KiMuLV− compared to KiMuLV+ or RaMuLV (Table 1). The greater efficiency of RaMuLV to replicate in S′L′ cells may be related to host range restrictions for MuLV growth in mouse cells (10). RaMuLV is NB-tropic, whereas KiMuLV is an N-tropic virus which would not be expected to replicate efficiently in B-type mouse cells, such as S′L′ cells (1, 10). However, S′L′ and 3T3FL cells have recently been shown to be equally sensitive to N- and B-tropic MuLV (8, 15), and host range restrictions would therefore not account for the differences in plaque titers between KiMuLV− and RaMuLV. Moreover, the present comparisons between KiMuLV− and its homologous, live virus revealed a 22-fold greater plaque titer for KiMuLV+ than KiMuLV− in S′L′ cells. These differences may be due to the inability of KiMuLV− to adsorb to, penetrate, or replicate effectively in S′L′ cells, although the present experiments do not distinguish between these alternatives. After KiMuLV− and KiMuLV+ were grown in identical host cells (3T3FL) for 12 weeks, the attenuated virus still produced comparatively lower titers of infectious progeny virus than KiMuLV+ (Table 1). Interfering particles were not demonstrated in the KiMuLV− population at the dilutions tested (Table 2). Assuming that such particles would interfere with the replication of coinfecting KiMuLV+, autointerference can be dismissed as an explanation for the poor in vitro infectivity of KiMuLV− (2). Finally, plaque formation by KiMuLV− in S′L′ cells exhibited a 1-hit dose response, suggesting that the basis for the poor infectivity of KiMuLV− is not the same as the restriction on the replication of infectious MuLV in a genetically nonpermissive mouse cell (Chart 1) (J. W. Hartley et al., unpublished data).

Some MuLV strains, when grown in tissue culture, have lost leukemogenicity rapidly without a concomitant loss of in vitro infectivity (16, 24, 33). KiMuLV− has been propagated for a long period in tissue-cultured mouse cells, which may explain its comparatively high degree of attenuation. We do not know the quantity of virus released by individual cells.
whether the attenuated virus was present in the original KiMuLV* stock that has been used to infect MEC1 cells (14, 26, 29). It is more likely that the attenuated virus developed during its in vitro passage by mutation or by recombination with endogenous viral genes (1, 30, 31).

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