Rescue of Murine Sarcoma Virus from a Sarcoma-Positive Leukemia-Negative Cell Line: Requirement for Replicating Leukemia Virus


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The nature of murine sarcoma virus (MSV) "defectiveness" was investigated by employing an MSV-transformed mouse 3T3 cell line which releases noninfectious virus-like particles. Rescue kinetics of MSV, observed after murine leukemia virus (MuLV) superinfection of these "sarcoma-positive leukemia-negative (S+L−)" mouse 3T3 cells, consisted of a 9- to 12-hr eclipse period followed by simultaneous release of both MSV and MuLV with no evidence for release of infectious MSV prior to the production of progeny MuLV. Addition of thymidine to the growth medium of MuLV-superinfected S+L− cells at a concentration suppressing deoxyribonucleic acid synthesis inhibited the replication of MuLV and the rescue of MSV. MSV production closely paralleled MuLV replication under a variety of experimental conditions. These results suggest that replication of MuLV is required for the rescue of infectious MSV from S+L− cells and that one (or more) factor, produced late in the MuLV replicative cycle, is utilized by both viruses during virion assembly. During the course of these experiments, virus stocks were recovered which contained infectious MSV in apparent excess over MuLV. These stocks were used for generating new S+L− cell lines by simple end point dilution procedures.

Certain ribonucleic acid (RNA) sarcoma viruses isolated from two well studied animal systems, avian and murine, have been shown to be defective in one or more functions required for virus replication. Envelope-associated properties of some sarcoma viruses are dependent upon coinfection with the corresponding leukemia virus. These properties include type-specific neutralization antigens, host range, and interference patterns (11, 14, 15, 21).

The murine sarcoma-leukemia virus system provides several advantages for the study of the relationships between sarcoma and leukemia viruses. Continuous lines of apparently virus-free, contact-inhibited mouse 3T3 cells have been developed which are as susceptible to murine sarcoma virus (MSV) infection as primary embryo cultures (3, 20). Quantitative assays for both MSV and murine leukemia virus (MuLV) have been developed by using the 3T3 mouse cell line (6, 15). Moreover, two distinct types of clonal cell lines of 3T3 mouse cells transformed by MSV in the absence of replicating MuLV have been established recently (1, 5). One type of MSV-transformed cell containing the sarcoma genome in the absence of replicating leukemia virus, the "sarcoma-positive, leukemia-negative (S+L−)" line, has been shown to release an apparently noninfectious type C virus-like particle (4) which may be analogous to the α-type Rous sarcoma virus (O) described in the avian system (11).

A second type of 3T3 cell containing the MSV genome in the absence of replicating MuLV has been isolated after transformation of BALB/3T3 cells by MSV. These MSV-transformed 3T3 cells, termed "nonproducer (NP)", differ from S+L− cells in that there is no evidence for the release of virus particles (2).

The present study was undertaken in an effort to further the understanding of MSV "defectiveness" and the corresponding role of leukemia helper virus by investigating the rescue of infectious MSV from the particle-releasing S+L− cells.
MATERIALS AND METHODS

Cell cultures. The S+L− cell line used for these studies was isolated from a semisolid agar colony and twice cloned as previously described (5). Mouse 3T3FL cells (5) were used for MSV assays. The cell line “C-182(5)”, a mixture of apparently normal 3T3FL cells and S+L− cells, was employed for MuLV assays (6).

McCoy's 5a medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum and antibiotics was used for growth and maintenance of all cell lines. Eagle's minimum essential medium fortified with 2X vitamins, 2X nonessential amino acids, 10% fetal calf serum, and antibiotics was used for all experiments involving 20 mm thymidine (18).

Virus. Moloney leukemia virus (MLV) was obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Md., as crude tissue culture fluid from MSV-infected JLSV9 cells (22). This preparation contained 5 x 10^3 focus-forming units (FFU) per ml and was used for the superinfection of S+L− cells.

A second preparation of MLV was obtained from University Laboratories, Highland Park, N.J., as a 10% spleen suspension from MLV-infected mice (9). This virus pool was used as a source of leukemia helper virus in MSV assays (5).

Virus assays. A rapid cell culture assay technique for MuLV, which is based on the induction of foci in S+L− cells after superinfection with MuLV, was employed in this study (6). A previously described modification of the Hartley and Rowe (15) MSV focus-forming assay system was used for MSV assays (5). Samples of virus for both the MuLV and MSV assays were prepared by freezing and thawing 3 times and supernatant fluids together followed by low-speed centrifugation to remove cell debris. These virus preparations were stored at −70°C prior to virus assays.

RESULTS

Kinetics of MSV rescue after superinfection of S+L− cells with MuLV. Some understanding of the nature of MSV “defectiveness” and the role of helper leukemia virus might be gained by a careful study of the kinetics of MSV release from S+L− cells after superinfection with MuLV. Medium containing 5 x 10^3 FFU of MuLV was added to 1-day-old cultures of 2 x 10^5 S+L− cells per 75-cm² flask. After an adsorption period of 1 hr, the monolayers were washed and fresh medium was added. At various intervals from 0 to 96 hr after adsorption, samples were harvested and assayed for both MuLV and MSV.

The resulting growth curves (Fig. 1) demonstrate that MSV production was first detected at 12 hr and that MSV titers thereafter increased logarithmically. These results are similar to the kinetics observed for Rous sarcoma virus rescue in the avian system (12).

Progeny MuLV could not be detected until 18 hr, although small amounts of MuLV replication before this time would not have been detected because of residual MuLV activity. After the initial 18 hr, MuLV production was in parallel with MSV and reached a plateau at 72 hr and then began to decrease. This early plateau and subsequent decrease in MuLV titers resulted in virus stocks which contained an apparent excess of focus-forming MSV over infectious MuLV (Fig. 1).

Thermal stability of MuLV and MSV. To determine whether the difference in the observed amounts of MSV and MuLV in virus stocks recovered from superinfected S+L− cells was a result of dissimilar thermal inactivation rates, 1-ml samples were incubated at 37°C, removed at intervals, and assayed for both MuLV and MSV. The inactivation rates were approximately the same, both being linear over a 24-hr period with an MSV half-life of 1.9 hr and an MuLV half-life of 2.1 hr. The MSV excess seen above is not, therefore, due to a more rapid thermal inactivation of MuLV.

Effect of fresh medium on virus production in MuLV-superinfected S+L− cells. Since the MSV excess seen above is not due to a more rapid thermal inactivation of the MuLV, the possibility exists that depletion of some growth factor selectively limits MuLV production. When cultures of MuLV-superinfected S+L− cells were left without a change of medium for 5 days, sharp declines in both MSV and MuLV titers were observed (Fig. 2). However, when the medium...
was changed daily from the fourth day on, the virus titers remained relatively constant with an apparent excess of MSV over MuLV.

End point titration method for isolation of new S+L— cells. The apparent 10-fold excess of MSV over MuLV in the 96-hr virus samples from the growth curve (Fig. 1) is surprising. All other studies have demonstrated MuLV titers to be higher than MSV titers (2, 15). Since two different assay systems are required to quantitate the two viruses, it is possible that the relative virus titers do not truly reflect the amount of infectious virus present. It is clear, however, that if MSV is in excess over MuLV, one should be able to obtain cells infected only by MSV simply by diluting beyond the leukemia virus end point. Because of the technical difficulties in observing single-hit sarcoma virus foci with 3T3FL cells (1, 5), the following experiment was devised to test the prediction that MSV could be isolated from MuLV by end point dilution.

A virus preparation obtained 96 hr after MuLV superinfection of S+L— cells was used to infect 3T3FL cells at limiting dilutions calculated to contain MSV in the absence of infective MuLV. To eliminate cultures infected with replicating MuLV, plates were individually subcultured weekly and monitored for replicating MuLV by assay of supernatant fluids. Duplicates of the remaining MuLV-negative plates were prepared, and one plate per set was challenged with helper MuLV. S+L— cells could be identified by this procedure since they did not produce MSV until superinfected with MuLV. The results (Table 1) show that 10 out of 17 plates infected with the $10^{-5.3}$ and $10^{-6.0}$ dilutions of the virus stock contained 3T3 cells infected with MSV, whereas only 6 of the 17 plates were positive for infectious leukemia virus. MSV-transformed cells present in the other plates may have been lost when the cells were passed.

Characteristics of new "S+L—" lines. The growth medium from the new S+L— isolates was repeatedly assayed for MuLV and MSV and has remained negative for over 10 passages. One cloned line has been obtained by using Microtest II plates (Falcon Plastics) at limiting cell dilutions (10). This clone has the characteristically transformed appearance of S+L— cells (5). Preliminary data indicate that the new S+L— cells have budding type-C particles (by electron microscopy) which incorporate tritiated uridine and band at 1.16 g/cm³ in sucrose gradients. Addition of MuLV to the new S+L— cell line results in the rescue of focus-forming MSV as before (4).

MSV rescue and the requirement for DNA synthesis. Since the MSV genome is already present in the S+L— cells, any requirement for DNA synthesis during the rescue of MSV would presumably reflect the requirement for intracellular establishment of MuLV (7, 8, 16, 18, 23). DNA synthesis in mammalian cells and also in MSV-transformed mouse cells has been shown to be effectively suppressed by the presence of excess

**Table 1. End point dilution method for isolation of sarcoma-positive, leukemia-negative (S+L—) cells**

<table>
<thead>
<tr>
<th>Virus dilution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of murine leukemia virus-positive plates/total</th>
<th>No. of murine sarcoma virus-positive plates/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-5.0}$</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>$10^{-6.0}$</td>
<td>4/8</td>
<td>7/8</td>
</tr>
<tr>
<td>$10^{-6.0}$</td>
<td>2/9</td>
<td>3/9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Virus prepared from murine leukemia virus superinfected S+L— cells harvested 96 hr after adsorption.

<sup>6</sup> A 0.2-ml amount of sample dilution adsorbed to each plate of 3T3 cells.
thymidine (0.02 M) in the growth medium (18). Control experiments demonstrate that, when S+L- cells were incubated in the presence of thymidine from 0 to 12 hr after superinfection, DNA synthesis was inhibited by 76% as measured by 32P incorporation. This effect was reversed by the removal of the excess thymidine, and vital staining of the cells revealed no evidence of cell death from the thymidine.

The requirement for DNA synthesis during the rescue of MSV was investigated by incubating S+L- cells in fortified Eagle's minimum essential medium with thymidine for the 12-hr period immediately prior to superinfection with MuLV and the 12 hr immediately after superinfection. All virus preparations were harvested 72 hr after infection and assayed for both MuLV and MSV. The results are summarized in Table 2. Under these conditions, the titers of MSV and MuLV were reduced by 99% when compared to titers in controls incubated without excess thymidine.

**DISCUSSION**

The isolation of S+L- cells and NP cells demonstrates that MSV can infect and transform mouse 3T3 cells in the absence of detectable MuLV (1, 5). To investigate the helper effect of leukemia virus and the nature of MSV defectiveness, it is of value to study MSV rescue from particle-producing S+L- cells because comparatively fewer functions may be required of leukemia helper virus for the production of biologically active MSV.

Kinetic studies of MSV and MuLV production (Fig. 1) after MuLV superinfection of S+L- cells revealed a common eclipse period and simultaneous production of the two viruses. This suggests that all or nearly all of the MuLV replicative cycle appears to be required for rescue of infectious MSV. The strikingly parallel growth curves indicate that some factor is constantly made available for the completion of infectious sarcoma virions at the same time it is utilized in the assembly of leukemia virions and that the viral genomes may compete for this factor.

If complete replication of MuLV is required for release of infectious MSV, then inhibition of MuLV replication in the S+L- cells should inhibit the production of infectious MSV. Treatment of S+L- cells with thymidine from 0 to 12 hr after MuLV adsorption resulted in a marked decrease in the production of both viruses (Table 2), indicating that MuLV infection and subsequent replication require DNA synthesis and that MSV rescue, in turn, requires the replication of MuLV. The fact that treatment of S+L- cells with thymidine prior to the addition of MuLV had no effect on the rescue of MSV indicates that the MSV genome in these cells appears insensitive to this inhibitor.

From the thymidine inhibition study, as well as from the growth curves, it can be seen that the titers of MSV rescued are closely related in each instance to the titers of MuLV replicated. This fact suggests again that MSV rescue requires complete MuLV replication.

One possible factor supplied by the replication of helper leukemia virus and required for the completion of both MSV and MuLV is envelope-associated antigen (2, 11, 17). The results of neutralization experiments in our laboratory indicate that S+L- cells, when superinfected with MuLV, release MSV which has acquired the type-specific envelope antigen of the superinfecting helper virus. It is unlikely, however, that envelope-associated antigen is the only missing function required for production of infectious MSV since the virus-like particles recovered from nonsuperinfected S+L- cells remain noninfectious, even after the adsorption-penetration barrier has presumably been by-passed by use of either cosedimentation procedures or infection in the presence of inactivated Sendai virus (4). This lack of infectivity may, in part, be explained by recent findings from this laboratory which indicate that the virus particles produced by S+L- cells are deficient in ribonucleic acid-dependent DNA polymerase activity (D. K. Haapala et al., manuscript in preparation). This deficiency in reverse transcriptase has also been found in Rous sarcoma virus α (O), the analogous noninfectious variant of Rous sarcoma virus (O) in the avian system (13, 19), and in apparently noninfectious virus particles from hamster tumor cells induced with a new strain of MSV (P. T. Peebles, et al., manuscript in preparation).

**Table 2. Effect of thymidine**

<table>
<thead>
<tr>
<th>Interval of exposure to thymidine (hr)</th>
<th>Virus titersb</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MSV (FFU/ml)</td>
</tr>
<tr>
<td>Absent (no MuLV added)</td>
<td>0</td>
</tr>
<tr>
<td>Absent</td>
<td>10^4±2</td>
</tr>
<tr>
<td>−12 to 0</td>
<td>10^4±6</td>
</tr>
<tr>
<td>0 to +12</td>
<td>10^5±8</td>
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a 0.02 M thymidine.

b Virus preparations obtained 72 hr after adsorption of 5 × 10⁴ focus-forming units (FFU) of MuLV to 5 × 10⁴ S+L- cells.
In the course of studying the kinetics of MSV rescue from S+L− cells, it was noted that at 96 hr after the addition of MuLV to S+L− cells MSV was present in excess over MuLV. This phenomenon is apparently not the result of a greater thermal inactivation of MuLV, nor of a selective depletion of a growth medium factor required primarily for MuLV production. Virus stocks with MSV excess over MuLV were used for the isolation of new S+L− cells by terminal dilution procedures (Table 1), and a new clone of S+L− cells obtained in this manner exhibited morphological characteristics of transformation similar to the initial S+L− cell line. This new S+L− clone releases noninfectious type C virus-like particles, a property identical to that of the original S+L− cell line (4). The MSV genome rescued from S+L− cells and used to prepare new S+L− cells appears, therefore, not to have been modified by helper virus, the rescue process, or host cell factors to the extent that it resulted in a reversion to an NP type of MSV cell (2).

Comparison of S+L− cells with NP cells, which also contain a stable MSV genome perhaps differing from that in S+L− cells, may provide new information regarding the essential contributions of leukemia helper virus required for MSV replication and, conversely, the minimum essential sarcoma virus genome needed for cell transformation.

Sarcomagenic viruses have not yet been isolated from human tumors, perhaps because some human tumors contain defective sarcoma genomes not detectable unless rescued by helper virus. It is, moreover, conceivable that antigenic but noninfectious virus-like particles, similar to the particles from S+L− cells, may be used to induce immunity without resulting in either infection or tumor formation.

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LITERATURE CITED


