Sarcoma-negative Leukemia-positive Transformed Cell Culture Established from a Murine Sarcoma Virus-induced Rat Bone Tumor

James C. Chan, James L. East, and Leon Dmochowski

Department of Virology, The University of Texas System Cancer Center at M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

SUMMARY

Inoculation of the Soehner-Dmochowski isolate of the Moloney strain of murine sarcoma virus (MSV), designated MSV-SD, consistently leads to the development of bone tumors in the susceptible New Zealand black (NB) rats. Two separate cell cultures have been established from 2 individual MSV-SD-induced NB rat bone tumors. Cells of 1 bone tumor culture, designated RBT-E, are in early in vitro passages. Cells form colonies in agar medium and take up 2-deoxy-D-[3H]glucose at a greatly enhanced rate, 5 times that of normal nontransformed rat embryo cells. Cells of the RBT-E culture release both MSV and murine leukemia virus (MuLV) and therefore contain sarcoma-positive leukemia-positive transformed cells. The other rat bone tumor culture, designated RBT-L, produced MSV at early passages. RBT-L culture has been passed over 130 times in vitro. Cells of the RBT-L culture form colonies in agar medium and take up 2-deoxy-D-[3H]glucose at an enhanced rate (3 times that of rat embryo cells), indicating the presence of transformed cells within the RBT-L culture. However, cells of the RBT-L culture at late passages (Passage 130 or more) produce only MuLV and no detectable MSV activity (as shown by the lack of tumor-inducing activity and the lack of focus-forming activities by direct assay or by infectious center assay). Attempts to rescue MSV activity from RBT-L cells by cocultivation with MuLV-producing mouse cells were not successful. The MuLV found in the RBT-L cells, however, is a competent helper virus capable of rescuing the MSV genome from MSV-SD-induced hamster bone tumor cells. All the available evidence supports the notion that late passages of the RBT-L culture contain transformed cells that do not produce conventionally detectable MSV. These cells are referred to as sarcoma-negative leukemia-positive cells.

The sarcoma-negative leukemia-positive cells represent a different kind of MSV-induced transformed cells and provide a unique system for studies in search of MSV markers such as MSV-specific antigens and MSV-specific nucleotide sequences.

INTRODUCTION

MSV preparations are known to contain a mixture of MSV and MuLV (3, 14). MSV can transform susceptible animal cells but cannot replicate by itself (2, 3, 6, 18, 20, 22), although exceptions to the latter point have been reported (4, 26, 27, 32). MuLV can replicate by itself, does not cause morphological transformation, and provides certain helper functions in aiding the replication of its defective MSV (14, 22, 28).

Among the MSV-transformed animal cells, several types of cells have thus far been isolated. These include: S+L+ cells (8); S+L- mouse cells (3, 5, 7, 17) from which MSV genome can be rescued; and S-L- revertant mouse cells from which no MSV genome can be rescued but which can undergo spontaneous retransformation (15, 25). Among the revertants of MSV-transformed cells, an S-L+ subtype has previously been described (16).

In previous publications (11, 31), we have reported the in vivo adaptation of MSV-M to induce bone tumors in newborn NB rats and the cell-free transmission of bone tumors. The virology, pathology, and histology of the MSV-induced rat bone tumors have been described (11, 30, 31). We report here a culture established from MSV-induced rat bone tumor that, after 130 or more in vitro passages, retains such transformation properties as refractile round cell morphology; the ability to form colonies in agar medium; and enhanced glucose uptake; and which produces MuLV but neither produces detectable MSV nor contains a resuable MSV genome.

MATERIALS AND METHODS

Induction of Rat Bone Tumor by MSV-SD

Soehner and Dmochowski induced bone tumors in 1 of 9 inoculated newborn NB rats with the MSV-M (30). From

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the bone tumors, a 20% CFE was made, and inoculation of this CFE into newborn NB rats led to the development of bone tumors in almost 100% of the inoculated newborn NB rats within a much shorter latent period (30). Since 1968, bone tumors have been successively transmitted in NB rats using CFE of the previous bone tumors. The MSV-M adapted to induce rat bone tumors was later designated as MSV-SD and was found to be antigenically similar to MSV-M (21).

Following i.p. inoculation of a 20% CFE of a rat bone tumor (in vivo serial passage 4), 10 of 10 inoculated newborn NB rats developed bone tumors in 16 days. A hind leg bone tumor from 1 of the rats was aseptically removed and set into culture. This culture has now been designated RBT-L.

MSV-SD has been serially passaged in vivo by inoculation of CFE of bone tumors, and the incidence of bone tumors has been consistently high (about 100%) (30). Four years after the establishment of RBT-L culture, bone tumors were induced in 6 of 6 newborn NB rats using CFE of a rat bone tumor at in vivo passage 27. The bone tumors of 2 hind legs of 1 animal were aseptically removed and set into culture to establish a line now designated RBT-E line.

Histology of Rat Bone Tumor from Which RBT-L Culture Was Established

The tumor was diagnosed as bone tumor. The overall histological description was cartilaginous islands interspersed with fibrous or fibrosarcoma areas. In some areas, a small amount of calcification is seen in spicules. There are large areas of cellular infiltration. A predominant feature is that of "malignant osteoid." Surrounding and emerging in many portions of the osteoid areas there exists a homogenous cell type consisting of spindle-shaped cells with pyknotic and vesicular nuclei arranged in irregular pattern. The vesicular nuclei contain multiple nucleoli. An infiltrate is observed, composed predominantly of round mononuclear cells with pyknotic nuclei and scant cytoplasm. In addition, many multinucleated giant cells are seen, some of which contain nuclei evenly distributed over the cytoplasm and some of which are oriented towards 1 side of the cells. Many of the mononuclear cells resemble myeloma cells with typical spoked wheel appearance. No polymorphonuclear leukocytes are seen.

Histology of Rat Bone Tumor from Which RBT-E Culture Was Established

The rat tumor was also diagnosed as bone tumor showing a histological appearance similar in most respects to the tumor from which the RBT-L culture was derived.

Media

The following media were used: APMEM; T-MEM; and modified McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% inactivated fetal calf serum and 100 μg/ml neomycin.

Tissue Cultures

**RBT-L Culture.** This cell culture was established from an MSV-SD-induced bone tumor (Tumor 1168) of the NB rat (13) and has been passaged weekly in T-MEM over 130 times. Previously, cell-free culture fluids of early passages (<Passage 40) of this RBT-L culture induced bone tumors in newborn rats (L. Dmochowski and R. L. Soehner, unpublished data). This indicates the presence of sarcoma virus at early passages. At the time of this study, the culture was already in excess of 130 passages and, due to a mechanical freezer failure, earlier passages were no longer available.

**RBT-E Culture.** This cell culture was established from another MSV-SD-induced bone tumor (Tumor 972) of the NB rat (11, 30) and has been passaged weekly in APMEM about 30 times.

**78Al WRC Cell Line.** This culture of rat embryo fibroblasts chronically infected with MSV-MuLV (8) was previously described (13).

**RE Culture.** This was a culture established from embryos of a pregnant NB rat and has been subcultured weekly in T-MEM for over 30 passages.

**NRK-2.** This cloned normal rat kidney cell line (12) has previously been described (13).

**S4-L Cell Subline (FG-10-0).** This culture was derived from a semisolid agar colony of MSV-transformed mouse cells (6, 7) and was supplied by Dr. Peter J. Fischinger of the National Cancer Institute. It was subcultured once a week in McCoy's Medium 5A.

**XC Cells.** The source of XC cells has previously been described (10). Cells of this line were subcultured weekly in T-MEM.

**BALB/3T3 Clone A31 Cells (33).** This culture was supplied by Dr. George Todaro of the National Cancer Institute, Bethesda, Md., and was subcultured weekly in McCoy's Medium 5A.

**Murine Sarcoma Virus Assays**

A direct focus-forming assay on NRK-2 or BALB/3T3 cells was carried out as previously described (13). Results were expressed as focus-forming units/ml.

An *in vivo* assay was carried out by inoculating newborn NB rats (3 to 7 days old) i.p. with 0.2 ml filtered (0.45 μm) virus preparation. Inoculated animals were observed for the development of bone tumors of limbs, spine, jaw, rib cage, or tail, which usually occurred within 2 months.

An infectious center assay was carried out as described by Aaronson and Dunn (1). RBT-E, 78Al WRC, and RBT-L cells were treated with Mitomycin C (25 μg/ml) for 1 hr at 37°, washed 3 times with growth medium, trypsinized, and counted. Cell dilutions (10⁴, 10³, or 10² cells) were added onto duplicate NRK-2 dishes (1 × 10⁵ cells/dish) preset 24 hr earlier in the presence of polybrene (2 μg/ml). Cell dilutions were also seeded onto empty dishes for determination of attachment efficiency of cells 24 hr later. Culture dishes were fed 2 days later and observed for the development of foci of transformed cells in 6 to 10 days.
Assays for MuLV

**XC Plaque Assay.** This assay was carried out essentially as described by Rowe et al. (29) using BALB/3T3 clone A31 cells. Dishes (containing 2 x 10^6 cells) were pretreated for one-half hr with polybrene (Aldrich Chemical Co., Milwaukee, Wis.) (8 µg/ml) to enhance virus absorption and were then infected with 0.2-ml virus dilutions. Two days after infection, the medium was changed. Four to 5 days after infection, each dish was overlaid with 5 ml T-MEM containing 1 x 10^4 XC cells. Three days after XC cell overlay, the dishes were fixed in absolute methanol and stained with Giemsa. Plaques were counted with the aid of an AO stereomicroscope.

**Focus Induction Assay.** This assay was carried out in S+L— (FG10-0) cells as described by Bassin et al. (7). Results were expressed as focus-inducing units/ml.

**Electron Microscopy**

RBT-L and RBT-E cells were prepared for electron microscopic examination according to the procedure previously described (21).

**Colony Formation in Soft Agar Medium**

Purified agar (Difco Laboratories, Inc., Detroit, Mich.) was dissolved in APMEM to 0.5% concentration. Five ml of 0.5% agar-APMEM were added per 60-mm Falcon plastic dish and allowed to solidify as a bottom layer. The cell suspension was mixed with 5 ml 0.5% agar-APMEM to a 0.33% final agar concentration. Dishes were incubated at 37° in 5% CO₂ and were refed by addition of 5 ml 0.33% agar-APMEM at weekly intervals. Colonies formed by transformed cells could be seen within 2 to 3 weeks.

**2-Deoxy-D-[3H]glucose Uptake**

This procedure was carried out essentially as previously described by Hatanaka and Gilden (19) and Hatanaka et al. (20). Cells were seeded in 60-mm Falcon plastic dishes at 8 x 10^6 cells/dish in T-MEM. Twenty-four hr after seeding, old medium was replaced with 4 ml prewarmed 1 times Hanks' BSS without glucose for 30 min at 37° and was then aspirated. Two ml of 2-deoxy-D-[3H]glucose (0.25 µCi/ml; specific activity, 450 mCi/mmol; Amersham/Searle, Arlington Heights, Ill.) in Hanks' BSS without glucose were added to each dish, and cells were incubated at 37°. After appropriate incubation periods (usually 15, 30, and 60 min), replicate plates were removed and were washed 3 times with ice-cold Hanks' BSS without glucose. Cells were scraped into 0.5 ml sterile distilled water with a rubber policeman and transferred to a small tube and dispersed using a Vortex mixer. An aliquot (0.1-ml) of the cell suspension was mixed with 0.6 ml NCS solubilizer (Amersham/Searle), and 5 ml toluene with omnifluor, (New England Nuclear, Boston, Mass.) were added; the radioactivity was determined in a scintillation counter. The rate of glucose uptake was expressed as uptake of radioactivity per mg protein. Protein was measured by the method of Lowry et al. (24).

RESULTS

**Type C Virus Particles.** Examination by electron microscopy revealed that type C virus particles were produced by both RBT-L and RBT-E cells.

**Colony Formation in Agar Medium.** When 10⁴ cells of each of the RBT-L and RBT-E cultures were plated in agar medium, distinct colonies were visible 19 days after plating. These were spherical clusters of cells, as can be seen in Fig. 1. No colonies were formed by normal nontransformed NRK-2 cells in the same experiment. Single agar colonies were isolated with a sterile Pasteur pipet under a microscope and were subcultured in 60-mm Petri dishes in liquid APMEM media. Each colony (from RBT-L or RBT-E) subsequently gave rise to a cell clone consisting of fusiform cells, some spindle-shaped cells, and many refractile round cells (Fig. 2). The number of round refractile cells increased in both RBT-L and RBT-E cultures after cloning in agar medium. When refractile round cells were isolated from the clone with a Pasteur pipet and trypsin-EDTA and plated onto another culture dish in liquid medium, colonies of biphasic cell morphology again appeared that consisted of refractile round cells growing above fusiform cells.

**Rate of 2-Deoxy-D-[3H]glucose Uptake by RBT-L and RBT-E Cells.** The rate of the glucose uptake by the 2 RBT cell cultures was compared with the normal nontransformed RE cell culture. Results summarized in Chart 1 show that the rate of glucose uptake by RBT-L cells was consistently 3 times that of normal RE cells, regardless of the incubation period. By comparison, the rate of glucose uptake by RBT-E cells was consistently 5 times that of the RE cells in the same experiment (Chart 1).

**Absence of Sarcoma Virus and Presence of Leukemia Virus in RBT-L Culture.** Culture medium supernatants of RBT-L and RBT-E cells were assayed for the presence of sarcoma and leukemia virus activities by in vitro assays. Results summarized in Table 1 show that both sarcoma virus and leukemia virus activities were found in the medium of 78A1 culture as well as in the RBT-E culture.

Fig. 1. Appearance of colony-formed by RBT-L cells in agar medium. × 100.
Virustiter
Sarcoma
Leukemia
Source of virus (FFU/ml)
(PFU/ml)
RBT-L
cells, passage 133
8.5 x 10^9
RBT-E
cells, passage 36.62
8.75 x 10^9
1 rat cell line, passage
9
6.1 x 10^9
Fig. 2. Biphasic appearance of agar medium-cloned RBT-L cells growing in liquid medium. Note the growth of refractile round cells above fusiform and spindle-shaped cells. x 300.

However, only leukemia virus activity and no sarcomagenic activity was found in the culture fluids of 2 different passages (passages 133, 138) of the RBT-L cell culture (Table 1). This lack of sarcomagenic activity was true whether NRK-2 or BALB/3T3 cells were used in focus-formation assay. Culture fluids of other passages (over passage 140) of the RBT-L cell culture and of several agar-medium-cloned RBT-L cell cultures have also been assayed in a similar manner, and results again confirmed the absence of demonstrable sarcoma virus activity. By infectious center assay, the virus in RBT-L culture (10^4 cells/dish) did not produce foci of transformed cells, whereas both the virus in RBT-E culture and the virus in 78A1 WRC culture (as few as 10^4 cells/dish) induced numerous foci of transformed cells (results not shown). The lack of detectable sarcomagenic activity in the RBT-L cell culture was further confirmed by other in vitro and in vivo assays, as will be shown.

Table 1
Comparative titers of sarcoma and leukemia viruses in RBT-L, RBT-E, and 78A1 cell cultures

<table>
<thead>
<tr>
<th>Source of virus</th>
<th>Sarcoma (FFU/ml)*</th>
<th>Leukemia (PFU/ml)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBT-L cells, pass 133</td>
<td>0</td>
<td>8.5 x 10^9</td>
</tr>
<tr>
<td>RBT-L cells, pass 138</td>
<td>0</td>
<td>3.4 x 10^9</td>
</tr>
<tr>
<td>RBT-E cells, pass 3</td>
<td>6.6 x 10^4</td>
<td>9.75 x 10^9</td>
</tr>
<tr>
<td>78A1 rat cell line,pass 90</td>
<td>6.1 x 10^9</td>
<td>1.2 x 10^7</td>
</tr>
</tbody>
</table>

* Focus-forming units in NRK-2 cells.
³ PFU in BALB/3T3 –XC plaque assay.
' MSV-M-producing Wistar rat cell line.

Presence of Sarcoma Virus in Different Passages of RBT-E Cell Culture. In contrast to the virus of RBT-L cells, transforming or sarcoma virus activity was consistently demonstrable in culture fluids of RBT-E cells at passages 3, 8, 13, and 26 (Table 2).

Lack of Detectable in Vivo MSV Activity by RBT-L Virus. Initially, experiments were carried out to test for the presence of MSV activity by assaying for tumor-inducing ability in susceptible NB newborn rats. Undiluted, filtered (0.45 μm) culture medium from either RBT-L or RBT-E cell culture was inoculated i.p. into 3- to 7-day-old newborn NB rats. Results summarized in Table 3 show that the culture fluid of RBT-E cells contained MSV, as evidenced by induction of bone tumors in 100% of the inoculated animals in 1.5 months. In contrast, the culture medium of RBT-L cells did not induce bone tumors in any of the 11 inoculated animals, even after 6 months.
cocultivated with an equal number of cells, producing only (0.45 μm) and assayed for any rescued sarcomagenic sarcomagenic virus (as assayed in NRK-2 cells), were hamster bone tumor cells (21), which do not produce any defective MSV could not replicate. In order to test for the rescue these questions pertains to the possibility that the MSV is present, but, because of a certain deficiency in the helper leukemia virus found in the RBT-L cell culture, the actual present, but, because of a certain deficiency in the leukemia viruses from both RBT-L cells is a competent helper virus. This was further confirmed by the rescue of MSV genome in S+L— (FG 10-0) cells (results not shown).

Competent Helper Virus in RBT-L Cell Culture. The lack of a demonstrable sarcoma virus in the RBT-L cell culture raises several questions as to the fate of the sarcoma virus presumably present at the time of tumor induction. One of these questions pertains to the possibility that the MSV is actually present, but, because of a certain deficiency in the helper leukemia virus found in the RBT-L cell culture, the defective MSV could not replicate. In order to test for the helper function of the leukemia virus found in RBT-L cells, rescue experiments were carried out. MSV-SD-induced hamster bone tumor cells (21), which do not produce any sarcomagenic virus (as assayed in NRK-2 cells), were cocultivated with an equal number of cells, producing only MuLV (IC-3T3 or RBT-L cells). Appropriate controls were also included (Table 4). Media were replaced 48 hr later, and cell lysates at 5 days after cocultivation were filtered (0.45 μm) and assayed for any rescued sarcomagenic activity on NRK-2 cells. Results summarized in Table 4 indicate that the leukemia viruses from both RBT-L cells and IC-3T3 cells were equally competent in rescuing a sarcoma virus genome from the MSV-SD-induced hamster bone tumor cells (Table 4). These results suggest that the leukemia virus in RBT-L cells is a competent helper virus. The competence of the MuLV as a helper virus that is present in RBT-L culture was further confirmed by the rescue of MSV genome in S+L— (FG 10-0) cells (results not shown).

In the same experiment, RBT-L cells were cocultivated with MuLV-producing IC-3T3 cells, but again no MSV was rescued from RBT-L cells (Table 4).

DISCUSSION

In this paper, evidence has been presented that the high-passage RBT-L cells that produced MSV during early passages no longer produce MSV in amounts detectable by such conventional assays as the direct focus-forming assay, the infectious center assay, and the tumor-induction assay. The high-passage RBT-L cells, however, still produce MuLV and exhibit several transformation properties.

The reason for the lack of detectable MSV in the high-passage RBT-L cells is not known. One feasible explanation is that MSV may be present in low concentration but that interference due to excess of MuLV in the RBT-L cells prevents the detection of MSV by the conventional assays described above. Levy et al. (23) previously reported that excess MuLV interfered with the focus formation by the Finkel-Biskis-Jinkins strain of MSV. In addition to reporting that the virus infectivity gradually decreased following serial passage of the MSV-MuLV complex at low dilution, Bondurant et al. (9) reported that MSV was much more sensitive to autointerference than was MuLV. They demonstrated that, after 5 serial passages of MSV-MuLV at low dilution, the titer of MSV was reduced by approximately 98%, whereas that of MuLV was reduced by less than 10% (9).

It is puzzling, however, that MSV could not be detected in RBT-L cells (Passages 133 and 138) when the MuLV titers were 8.5 × 10⁴ and 3.4 × 10⁴ PFU/ml, respectively (Table 1). These MuLV titers are comparatively lower than that of MuLV in RBT-E cells (9.75 × 10⁷ PFU/ml) and that of MuLV in 78A1 WRC cells (1.2 × 10⁸ PFU/ml) (Table 1). In spite of the much higher titers of accompanying MuLV (as determined by the XC plaque assay), the MSV was still detectable in RBT-E cells and 78A1 WRC cells by the direct focus-forming assay, while no MSV could be detected in RBT-L cells in the presence of lower titers of MuLV. The reasons for this are presently unknown.

Likewise, it should be pointed out that, in the rescue experiment (Table 4), cocultivation of HBT cells and the RBT-L cells led to the rescue of MSV that was detectable by the direct focus-forming assay. The results suggest that, if excess MuLV were present in the RBT-L cells, such
MuLV did not suppress focus formation by the MSV rescued from HBT cells.

Another possible explanation for the lack of detectable MSV in the high passage RBT-L cells can be entertained. As a result of prolonged in vitro passages of the RBT-L cells, progeny MSV have become defective in their transformation functions and therefore cannot be detected by the conventional assays described in this communication. Based on current data, no distinction can be made between these 2 explanations.

Direct evidence for the MSV informational role in the high-passage RBT-L cells will be difficult to obtain until more sensitive immunological and biochemical probes are available for detecting sarcoma-specific proteins and nucleic acids in MSV systems consisting of sarcoma viruses and large excesses of leukemia viruses.

The RBT-L cell line may serve as an interesting and useful model for studies of MuLV interference or autointerference, for studies on the defectiveness of the transformation function of MSV, and for studies in search of MSV markers, such as MSV-specific antigens and MSV-specific nucleic acids. Hopefully, through the development of MSV-specific immunological and biochemical probes, the informational role of MSV in the high-passage RBT-L cells may be resolved.

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