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Murine Sarcoma Virus Transformation of BALB/3T3 Cells: Lack of Dependence on Murine Leukemia Virus*

Stuart A. Aaronson,† John L. Jainchill, and George J. Todaro

VIRAL CARCINOGENESIS BRANCH, NATIONAL CANCER INSTITUTE, BETHESDA, MARYLAND

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Abstract. Murine sarcoma virus induces foci of morphologically altered cells in BALB/3T3 cultures. Focus formation in mouse cells has been thought to require the presence of a helper, murine leukemia virus, which is present in murine sarcoma virus stocks but by itself does not induce any morphological transformation of mouse cells. The present studies show that early after infection, the titration pattern for murine sarcoma virus in BALB/3T3 cells is "two-hit" since only foci produced by virus spread can be detected. Such foci require the presence of both viruses in the initially infected cell. By seven days the titration pattern is "one-hit" under culture conditions which allow the growth and detection of small foci of transformed cells induced by murine sarcoma virus alone. The "two-hit" titration pattern results from the inability to detect these foci. We conclude that murine sarcoma virus is able to transform mouse cells without requiring murine leukemia virus.

Introduction. Several systems to study cell transformation with oncogenic viruses have been described. With polyoma and simian virus 40 (SV40), using either the colony transformation method or the agar growth method, the number of transformants produced is proportional to the virus concentration.[1] This "one-hit" relationship between virus dilution and transformed foci has also been found with the RNA-containing tumor virus, Rous sarcoma virus.[2] With each of these viruses, a single infectious unit is sufficient to produce transformation in a system that allows for its detection.

With murine sarcoma virus (MSV), Hartley and Rowe[3] and others[4,5] have described a different pattern for focus formation. Here, the number of foci that are observed falls as the square of the virus dilution (a "two-hit" titration pattern). The addition of optimal levels of "helper" murine leukemia virus (MuLV), which itself does not produce morphological alteration, changes the titration pattern from two-hit to one-hit. The two-hit pattern has led to the conclusion that murine sarcoma virus, unlike the avian sarcoma virus, is defective and requires helper leukemia virus for successful focus formation. More recently, Parkman *et al.*[5] have made the interesting observation that the same stock of murine sarcoma virus which gives an apparent two-hit pattern in mouse embryo cells, gives a one-hit pattern in rat cells, which suggests that properties of the host cell can influence the results of a focus formation assay.

In previous studies we have described transformation assays using continuous lines of mouse cells, BALB/3T3 and NIH/3T3.[6] Under our conditions, using murine sarcoma virus stocks produced in tissue culture and filtered to remove aggregates, focus formation has always shown a one-hit pattern.[6,7]

A clear demonstration that the sarcoma virus is not defective for transformation in mouse cells came from the recent finding that MSV-transformed mouse cell clones could be isolated that produce neither MSV nor MuLV but from which the MSV genome could be rescued by the addition of MuLV.[8] In the present paper experiments are described which may resolve some of the apparent contradictory results previously obtained concerning the interaction between murine sarcoma virus and mouse cells.

Materials and Methods. Cell culture: Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum in 50 mm plastic Petri dishes (Falcon Plastics, Los Angeles). Cells were subcultured with 0.1% trypsin in phosphate-buffered saline. The continuous mouse embryo cell line, BALB/3T3, was used; its sensitivity to the growth of MSV and MuLV is similar to that of primary BALB/c embryo cells.[6] Two MSV transformed BALB/3T3 clonal lines, MSV-BALB/3T3-4[7] and MSV-8 clone 1[8] were also used. The former releases both high titered MSV and MuLV into the culture fluid while the latter, although it contains the murine sarcoma virus genome, releases no detectable MSV or MuLV. The virus released by MSV-BALB/3T3-4 is a BALB-tropic pseudotype of the Moloney strain of MSV [Moloney MSV (BALB/c T1)][8] titering 5×10^4 focus-forming units/ml. This pool contains a 20-fold excess of BALB-tropic MuLV. A Rauscher pseudotype of Moloney MSV was obtained by superinfection of MSV-8 clone 1 with Rauscher leukemia virus. This MSV pool titered 10^5 focus-forming units/ml, contained a 15-fold excess of Rauscher MuLV, and had the host range and antigenic properties of the Rauscher MuLV.[8] Rauscher MuLV was obtained from Dr. F. Rauscher (NIH).

Virus assays: All virus preparations were passed through a 0.22 μm membrane filter (Millipore Co.) immediately prior to use. In general we have found that with tissue culture-produced virus there was little loss of activity after filtration. In contrast, virus prepared from tumor extracts by the Moloney procedure[9] or from tissue culture cell lysates showed a marked loss of activity after filtration. Presumably the latter preparations contain a large proportion of virus aggregates and virus associated with cell debris.

Focus formation by MSV and growth of MuLV were assayed on BALB/3T3 cultures inoculated with $1\text{–}2 \times 10^5$ cells per Petri dish 24 hr prior to infection. Cells were first treated for 1 hr with 4 ml of medium containing 25 μg/ml of DEAE-dextran (mol wt more than 2×10^6, Pharmacia Co.).[10] Cultures were exposed to 0.5 ml of virus for 1 hr with frequent shaking, and then fresh medium was added. The medium was changed once on the fourth or the fifth day after infection, and the final scoring of MSV foci was at 7 days.

MuLV was titered using the XC plaque test.[11] This test is based on the observation by Klement *et al.*[12] that in the presence of MuLV-infected mouse cells, a Rous sarcoma virus-induced rat tumor line, XC, undergoes synctium formation. XC cells were inoculated at 10^6 cells per Petri dish on the seventh day after infection of mouse cells. Three days later cultures were fixed with methanol for 30 min and then stained with Harris hematoxylin for 45 min. The MuLV-producing foci were seen as plaques. This method gave comparable titers to those obtained by the complement fixation method[13] for the MuLV preparations used in these studies.

Results. The finding that MSV-transformed foci could be isolated that contained no detectable leukemia virus and none of sarcoma-leukemia virus associated group-specific antigens[8] suggested that MSV was capable of transforming

mouse cells without requiring the continued presence of infectious MuLV. The presence of MuLV was, however, necessary for the release of infectious MSV. It seemed likely, therefore, that focus formation was dependent upon two mechanisms. If an MSV particle and an MuLV particle infected the same cell, progeny MSV would be formed. This would result in the rapid development of a focus caused primarily by the spread of MSV to adjacent cells. If, however, MSV transformed a cell in the absence of MuLV, the formation of the focus would be dependent entirely upon cell division of the originally infected cell. If this hypothesis were true, it should be possible to separate the two mechanisms by assaying focus formation early (3 days) and later (7 days) after infection. At an early time, it should not be possible to recognize those foci caused solely by cell division. Only those foci produced primarily by spread of infectious virus should be detectable.

The titration patterns of an MSV stock (Moloney MSV/BALB/c T1) at 3 and 7 days are plotted in Figure 1 in the manner proposed by Hartley and Rowe.[3] By this method, a linear titration pattern gives a straight line parallel to the X axis while a two-hit curve falls at a 45° angle. At 3 days, the titration pattern for MSV was clearly two-hit, indicating the requirement for two viruses infecting the same cell in order for expression of a focus. In marked contrast, by 7 days the titration pattern was linear in the *same* plates. In further experiments with the Rauscher pseudotype of Moloney MSV, similar results have been obtained. In a parallel assay performed with the addition of an optimal multiplicity of Rauscher MuLV (0.1–0.3 plaque-forming units/cell), a one-hit pattern was seen at 3 days. Thus the presence of MuLV in nearly every cell allowed the early detection of essentially all of the MSV added at each dilution tested; by day 7 the titration pattern was still linear but at a level 2–3 times higher than that seen at 3 days.

Time course of focus formation: The time course of appearance of MSV foci was studied in cultures inoculated with 10 or 100 focus-forming units of MSV per culture in the presence or absence of added "helper" leukemia virus (Fig. 2). With the higher MSV dose, in the absence of added MuLV, only 10 foci were observable by 3 days. In parallel Petri dishes also infected with helper virus, the number of foci seen at 3 days was much higher, averaging 65 foci per plate. Here, the number of foci increased to about 150 foci per plate by 7 days compared to about 100 foci in Petri dishes not exposed to excess MuLV. By using the lower dose of MSV in the presence of added MuLV, we observed five foci at 3 days, and this number increased at a rate roughly parallel to that seen for helper virus-infected cultures at the higher MSV dose. In the absence of MuLV, however, no foci could be seen in Petri dishes until as late as 5–6 days, and only by 7 days had the number of foci reached an average of six foci per plate. These were generally smaller in size, and some contained as few as 4–8 round, refractile, or spindle-shaped cells. Such foci could, however, easily be distinguished from the surrounding normal BALB/3T3 cells (see Fig. 4*A*).

Reconstruction experiments: The studies so far were consistent with the hypothesis that early after infection the observable foci were caused primarily by virus spread while the smaller foci appearing at later times were either the

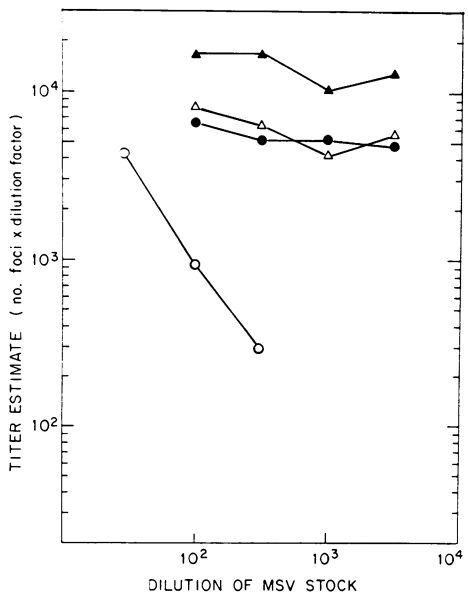

FIG. 1.—Infection of BALB/3T3 with serial dilutions of MSV. Horizontal direction of curve indicates one-hit kinetics.[3] Titration pattern at 3 days (○) and at 7 days (△); no added MuLV. Titration pattern at 3 days (●) and at 7 days (▲); 0.1 plaque-forming unit/cell of MuLV added.

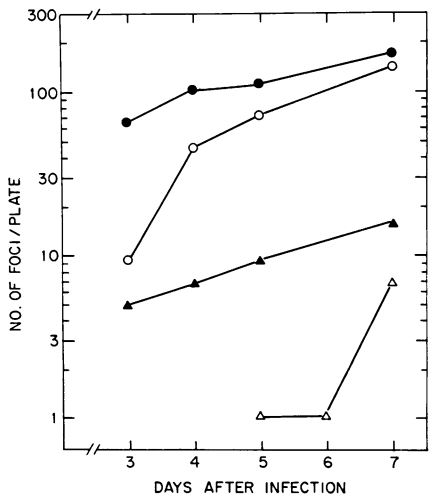

FIG. 2.—Time course of focus formation by MSV in BALB/3T3 cells. Each point represents the average number of foci in 3 Petri dishes. 100 focus-forming units of MSV were inoculated with (●) or without (○) helper MuLV. 10 focus-forming units of MSV were inoculated with (▲) or without (△) helper MuLV.

result of cell division of the initial transformant or caused by secondary foci from MSV spread. To test the extent of secondary focus formation a reconstruction experiment was performed. Thirty MSV-transformed cells releasing both MSV and MuLV were mixed with 2×10^5 normal BALB/3T3 cells and inoculated into each of several new Petri dishes. The time course for recognition of foci is shown in Figure 3*A*. With or without added helper MuLV the number of foci seen at 3 days was similar (27 foci per plate). These foci were quite large, containing as many as 50–100 cells. Figure 3*A* shows that the number of foci seen at 7 days was greater than the number of sarcoma cells inoculated. This was especially pronounced where high levels of leukemia virus were also present. In the reconstruction experiment, the increase in MSV foci in the helper-infected cultures over the number seen without added helper must have been caused by secondary infection. This is also the most likely explanation for the parallel but 2- to 3-fold higher titration pattern seen for MSV in the presence of optimal helper in Figure 1.

In Figure 3*B* the kinetics of focus formation are shown for BALB/3T3 cultures (2×10^5 cells) inoculated in a reconstruction experiment with about 100 nonproducer cells (MSV-8 clone 1). In the absence of added MuLV, very few foci were observable even by 5 days, and those that were visualized consisted of only

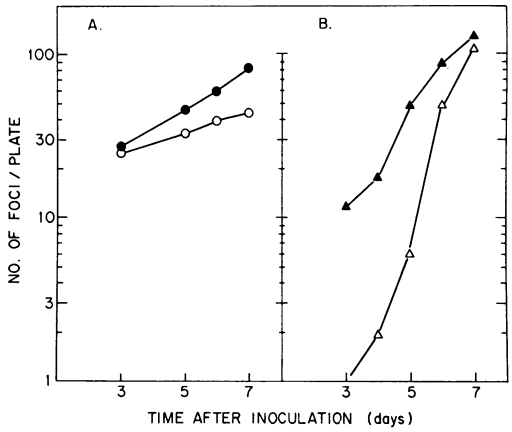

FIG. 3.—Time course of focus formation by MSV-transformed producer and nonproducer cells. (*A*) Reconstruction experiment in which 30 transformed cells releasing both MSV and MuLV were inoculated along with 2×10^5 BALB/3T3 cells into Petri dishes with (●) or without (○) added helper MuLV. (*B*) Reconstruction experiment in which 100 nonproducer MSV-transformed cells were inoculated along with 2×10^5 normal BALB/3T3 cells with (▲) or without (△) added helper MuLV.

4–8 cells. By 7 days about 100 foci were detectable, and the number of transformed cells per focus ranged from 10 to 30 cells. Some large MSV foci were detected as early as 3 days in the presence of added MuLV, although not nearly as many as the number of MSV-transformed cells added. In this test then not every MSV nonproducer cell had the genome in a readily rescuable form. By 7 days, however, in the presence of leukemia virus the number of foci increased to over 100 per plate. At this time, the foci were much larger than foci in the Petri dishes containing nonproducer cells without added helper virus.

Figure 4 shows examples of MSV foci in BALB/3T3 cells. A small focus of

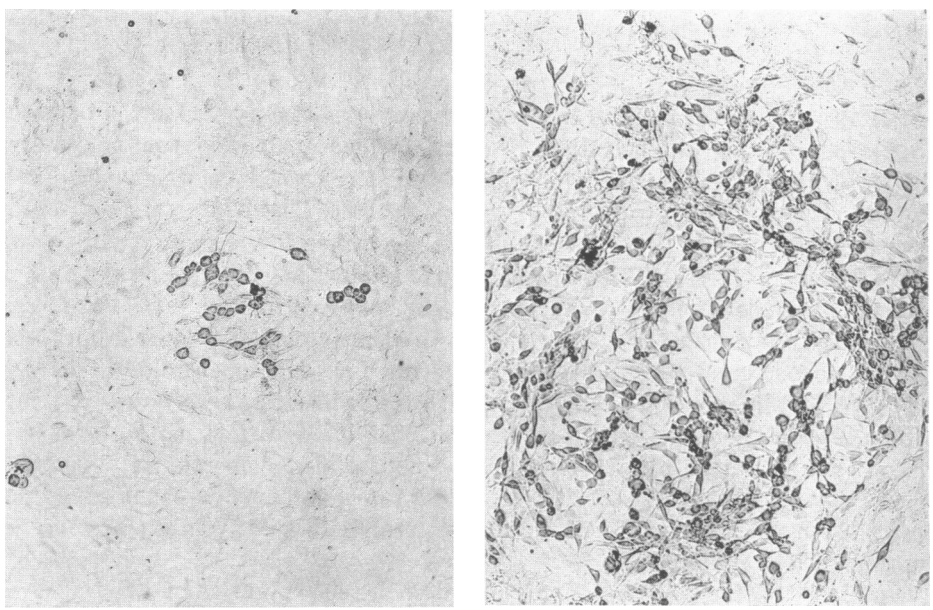

FIG. 4.—Transformed foci induced in BALB/3T3 cells by MSV. On the left is a small focus seen at 7 days after infection. On the right is a much larger focus 5 days after infection. ×100.

rounded and spindle-shaped, highly refractile cells is seen in Figure 4A. This is typical of the small foci that appear late (7 days) in MSV-infected cultures. Figure 4B shows a much larger focus 5 days after infection; such a focus obviously could not have resulted solely by cell division of the initial transformant.

Transformation assay with MSV: The MSV transformation assay can be performed in a manner strictly analogous to that used for oncogenic DNA viruses.[1] In this experiment a Petri dish containing 1×10^6 cells was infected with 5×10^3 focus-forming units of MSV (10^5 MuLV). After the adsorption period, decreasing numbers of acutely infected cells were plated, and the transformation frequency was determined after the cells grew into colonies by counting the number of transformed colonies at cell dilutions where convenient numbers could be scored (5–100 transformed colonies per plate). The number of transformants was directly proportional to the number of infected cells plated (Table 1). The calculated focus-forming titer was greater with the transforma-

TABLE 1. *Transformation assay with MSV.**

No. cells inoculated per plate	No. transformed colonies per plate	Transformation frequency (%)†	Calculated focus-forming units‡
2×10^5	> 500, > 500	—	—
1×10^4	119, 127, 131	1.3	1.3×10^4
1×10^3	10, 16, 17	1.4	1.4×10^4
1×10^2	0, 1, 3	1.3	1.3×10^4

* 0.5 ml containing 5×10^3 focus-forming units of MSV and 10^5 plaque-forming units of MuLV were added to 10^6 BALB/3T3 cells for 3 hr at 37°C with frequent shaking (every 15 min). Cells were transferred 24 hr later to new Petri dishes at different cell dilutions for the transformation assay. Transformed colonies were scored at 16 days.

† The number of transformed colonies/number of cells inoculated.

‡ Transformed colonies/cells plated × cell dilution.

tion assay than with the focus-forming assay. This may be because more cell divisions were allowed for full expression of the transformed state. When 100 cells were plated, individual colonies grew up, some of which were clearly recognized as transformed. These had to arise as the result of cell division of the original sarcoma virus-infected cell.

Discussion. Cytolytic viruses are generally assayed under conditions where the indicator cells are confluent; this favors the spread of virus from cell to adjacent cell thereby allowing the rapid enlargement of the plaque or the focus. In contrast, assays for the transforming effect of viruses are more nearly optimal when rapidly dividing cells are infected and several cell divisions are allowed subsequent to infection for optimal expression of the transformed state. The assay systems used to study the viruses of the murine sarcoma-leukemia complex have been closer to the former than the latter and have, therefore, emphasized the infectious properties of the viruses rather than their transforming properties. In this paper we have approached the study of the biological effect of the sarcoma virus by methods that have been used for the study of other transforming viruses. The present studies show that the Moloney strain of MSV is able to transform mouse cells without requiring leukemia virus.

Two classes of MSV-transformed cells have been described.[8] The first and most common releases large amounts of both MSV and MuLV, with the latter

in excess. This class of transformants results from dual infection by MSV and MuLV. Another class of MSV transformants, presumably infected only with MSV, has been recognized that releases neither virus although the cells contain the sarcoma genome. In the standard MSV assay, foci that produce infectious virus rapidly enlarge by spread of MSV to neighboring cells, while those that are not producing virus enlarge much more slowly. The detection of this latter class requires optimal culture conditions, including rich medium and hardy cells as well as enough cell divisions for expression of the transformed state. The use of homogenous populations of contact-inhibited cells further facilitates the detection of small foci. The foci produced by dual infection, on the other hand, are easy to recognize in any assay. In the pure transformation assay, however, where cell division is the primary mode of focus enlargement, transformed colonies of either type are not recognizably different in size.

In assay systems which only recognize large foci associated with spreading virus an apparent one-hit titration pattern can be obtained by the use of preparations that are aggregates of MSV and MuLV. Aggregates form when virus stocks are prepared from tumors by the Moloney procedure or when tissue culture-grown stocks of MSV and MuLV are cosedimented.[14] Another source of apparent one-hit kinetics occurs when the MuLV is in such large excess to the MSV that there is a very low probability that any cell will be solely infected by MSV. None of these factors apply to the present experiments.

Murine sarcoma virus transformation is similar in several ways to SV40 transformation. Both viruses can transform but by themselves are not able to complete their growth cycle in mouse cells. With SV40, the virus can be rescued from transformants by cocultivation with monkey kidney cells[15] indicating that the whole genome is present. In MSV-transformed nonproducer BALB/3T3 cells, the entire genome must be present, since it can be rescued from clones many generations after the transforming event by the addition of leukemia virus.[8] With MSV, at least one cell division is necessary subsequent to infection in order for fixation of the transformed state to occur.[16] This requirement has also been shown for transformation by DNA-containing tumor viruses.[17] In SV40-transformed cells the genome persists by covalent linkage to the host DNA. The MSV genome may well persist in the transformed "nonproducer" cell in the same way. An RNA-dependent DNA polymerase[19] could put the sarcoma virus information into a form that allows it to become integrated.[20]

Antibodies produced to SV40 tumors in one species react by complement fixation or fluorescent antibody methods with SV40-infected or transformed cells of any species. With MSV, however, no reactivity has so far been detected,[8] although sera from a number of MSV-tumored animals have been tested against nonproducer mouse transformed cells. The fact that antisera to MSV/MuLV fail to react with the nonproducer cells indicates that these antisera detect viral antigens and not virus-specified cellular antigens. Whether MSV does induce formation of virus-specified cellular antigens in analogy to the T-antigen induced by SV40 is currently being investigated.

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† Requests for reprints may be addressed to Dr. S. A. Aaronson, Viral Carcinogenesis Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014.

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