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Chemical Induction of Focus-Forming Virus from Nonproducer Cells Transformed by Murine Sarcoma Virus

(helper virus/murine leukemia virus/murine sarcoma virus/focus-forming units)

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ABSTRACT **Focus-forming virus can be induced by chemicals from virus-negative clonal lines of cells transformed by murine sarcoma virus. The viruses activated from such nonproducer cells have the host range and serologic properties of endogenous helper viruses of the cells rather than those of the sarcoma virus used to transform them. The evidence indicates that murine sarcoma virus-transformed nonproducer cells of two species contain the genetic information for both murine sarcoma and helper virus production in an unexpressed form.**

Many studies have suggested that murine RNA tumor viruses are transmitted vertically and can be activated *in vivo* under various conditions. In tissue culture, virus-negative BALB/c and random bred Swiss mouse embryo cells were shown to "spontaneously" make murine leukemia virus (MuLV) after prolonged periods of cultivation (1). These findings raised the possibility that the genetic information for virus production might be present in an unexpressed form in each cell. Recently, chemicals such as 5-bromodeoxyuridine have been shown to efficiently activate C-type viruses that do not form foci from clonal cell lines of AKR (2) and BALB/c (3) mouse embryo cells. In BALB/c cells, the C-type virus induced is difficult to study because it replicates very poorly in the cells from which it is activated.

Lines of cells that are transformed by murine sarcoma virus (MSV) have been isolated; these cells produce no detectable virus and contain no measurable viral antigens (4–6). Such nonproducer cells release MSV when superinfected with helper leukemia virus. The envelope properties of the rescued MSV genome are those of the leukemia virus used to rescue it. The present report demonstrates that MSV-transformed nonproducer cells can be induced by chemicals to make a focus-forming virus. The host range and serologic properties of MSV that are induced can differ markedly from those of the virus initially used to transform the cells. The findings indicate that the genetic information for both MSV and an endogenous helper virus exist, in the absence of virus production, in rat and mouse nonproducer cells transformed by MSV.

METHODS

Cells were grown in Dulbecco's modification of Eagle's medium. Many of the lines have been described elsewhere and include the continuous mouse cell lines BALB/3T3 (7) and NIH/3T3 (8), and the rat NRK line (9). Nonproducer clonal lines of BALB/3T3 cells were transformed by the Moloney (M) strain of MSV, BALB/3T3 (M-MSV) (5) and by the Kirsten (Ki) strain of MSV, BALB/3T3 (Ki-MSV) (6). A KiMSV transformed nonproducer clone of NRK cells, NRK (KiMSV), was also studied (6). The viruses used here included Rauscher MuLV and Ki-MSV (Ki-MuLV). MSV was titrated by a focus-forming assay (5), while helper MuLV was assayed by the XC plaque test (10). The complement-fixation assay for antigens of the murine sarcoma-leukemia complex has been described (11). Virus neutralization assays were performed by a focus-reduction method (5). Assay for supernatant viral polymerase, a method for detecting C-type virus replication and transmission, is described in detail (ref. 3; Aaronson and Scolnick, in preparation).

Chemicals used included 5-bromodeoxyuridine (BrdU), 5-iododeoxyuridine (IdU), 5-iododeoxycytidine (IdC), 5-fluorouracil, and 2-amino-6-mercaptopurine (Calbiochem, Los Angeles, Calif). Cytocholasin B was obtained from Imperial Chemical Industries Research Labs, Cheshire, England.

RESULTS

Time course of induction of focus-forming virus from MSV nonproducer cells

It has recently been shown that BALB/3T3 cells and a large number of other clonal cell lines derived from BALB/c mice can be induced to make C-type viruses that do not form foci (3). The induced virus contains viral-specific reverse transcriptase and can be transmitted to new mouse cell cultures. Bromodeoxyuridine (BrdU), which initially was found to efficiently activate MuLV from AKR cells (2), was used to study induction of virus from MSV nonproducer cells. About 5×10^4 BALB/3T3 (Ki-MSV) cells were exposed to 100 μg/ml of BrdU for 24 hr. As shown in Fig. 1, focus-forming virus was readily detected in culture fluids by 3 days and reached a peak of 5×10^2 FFU/ml at 7 days. Afterwards, the titer of MSV decreased so that after 15 days, virus was no longer detectable. A similar time course of induction was observed with a clone of BALB/3T3 (M-MSV) cells. These results compare favorably with the time course of induction of viruses, which contain viral polymerase and do not form foci, from normal BALB/3T3 cells (3).

Focus-forming virus was also activated by treatment of Ki-MSV-transformed nonproducer NRK cells with BrdU. Here transmissible MSV was detectable as early as 2 days,

Abbreviations: MSV, murine sarcoma virus; M-MSV, Moloney-MSV; Ki-MSV, Kirsten-MSV; R-MuLV, Rauscher murine leukemia virus; FFU, focus-forming unit(s).

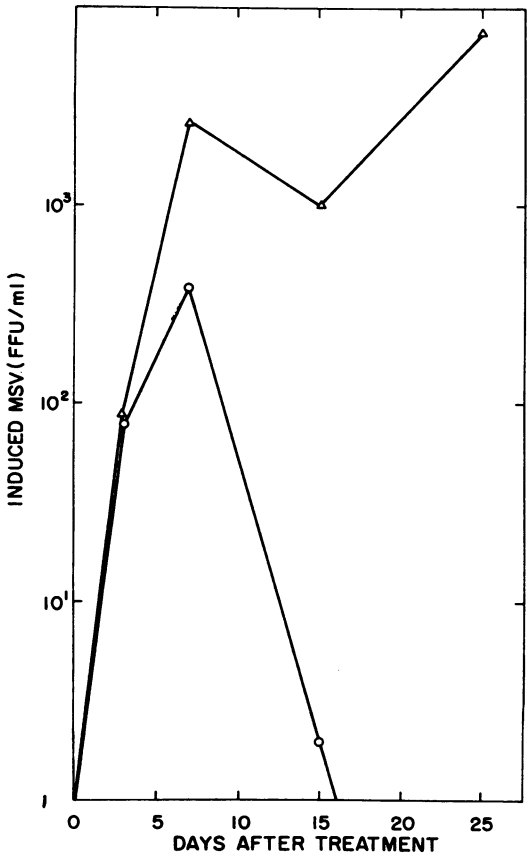

FIG. 1. Induction of focus-forming virus from nonproducer BALB/3T3 (Ki-MSV) cells. 5 × 10^5 nonproducer cells were exposed to 100 μg/ml of BrdU for 24 hr and then transferred at a concentration of 10^5 cells per plate either to empty Petri dishes or to Petri dishes containing 10^5 NIH/3T3 cells. The time course of MSV induction from BrdU-treated BALB/3T3 (Ki-MSV) cells alone (O) or cocultivated with NIH/3T3 cells (Δ) is plotted.

reached peak levels at around 7 days, and declined to below detectable titers by 3 weeks. Treatment with BrdU for 24 hr resulted in a broad dose-response curve for induction of MSV. The range was from 10 to more than 2000 μg of BrdU/ml of culture medium, with the peak for induction at around 200 μg/ml. Exposure of cells to BrdU for longer than 24 hr resulted in significant cell toxicity.

In previous studies, it was observed that helper MuLV, induced "spontaneously" or by chemicals from BALB/c cells, grew much better in NIH/Swiss than in BALB/c embryo cultures (1, 3); the viruses thus fit into the class of N-tropic murine leukemia viruses (12). Around 10^5 BALB/3T3 (Ki-MSV) cells were subcultured on the day after treatment with BrdU with growing NIH/3T3 cells. Under these conditions, as shown in Fig. 1, MSV production persisted. Around 200–1000 nonproducer cells that were treated with BrdU could be cocultivated with NIH/3T3 cells and still release detectable focus-forming virus. In contrast, cocultivation of as many as 10^6 untreated nonproducer cells with NIH/3T3 cells did not result in activation of MSV.

Host range and serologic properties of induced sarcoma viruses

The host ranges and serologic properties of viruses activated from different MSV nonproducer cell lines were compared. The host range of the virus activated from a BALB/3T3 (M-MSV) clone was identical to that from a BALB/3T3 (Ki-MSV) line (Table 1). Yet, the host ranges of the two MSV strains that were used to transform these cells were very different (5, 6). Culture fluids of BALB/3T3 (Ki-MSV) cells, which were treated with BrdU and then cocultivated with NIH/3T3 cells, were tested for XC plaque formation. Small, poorly defined XC plaques were observed in NIH/3T3 but not in BALB/3T3 cultures.

The host ranges of the focus-forming viruses induced from the BALB/3T3 (KiMSV) and NRK (Ki-MSV) cell lines were different, although the cells had initially been transformed by the same preparation of Ki-MSV (Ki-MuLV). MSV induced from BALB/3T3 cells that were transformed by Ki-MSV transmitted almost equally well to NRK and NIH/3T3 cells, while MSV activated from NRK (Ki-MSV) cells produced foci only on NRK cells. Focus-formation by the latter MSV was not inhibited by either AKR rat or Friend–Moloney–Rauscher neutralizing sera, sera which inhibit almost all known mouse C-type viruses. Under the same conditions, focus-formation by Ki-MSV (Ki-MuLV), which was the source of the MSV genome in the nonproducer NRK cell line, was completely inhibited by ARK antiserum. The above results indicate that the host range and serologic characteristics of the induced focus-forming viruses reflect those of endogenous helper viruses and are independent of the MuLV pseudotype of MSV with which the cells were originally transformed.

Properties of the induced sarcoma genome

It has been shown that the M-MSV and Ki-MSV genomes present in cells that do not produce MSV are capable of transformation but require MuLV for virus production (5, 13). It was possible that the induced MSV might acquire the ability to replicate as a single infectious virus. In that event, each MSV focus selected should release infectious MSV. NRK and NIH/3T3 cultures were infected with MSV that had been activated from BALB/3T3 (Ki-MSV) cells. At around 7 days, MSV foci were isolated by means of cloning cylinders from Petri dishes containing one or at most two foci. As shown in Table 2, out of a total of nine such focus-derived lines that were examined, none showed evidence of transmissible MSV or detectable reverse transcriptase activity in supernatant fluids. Furthermore, none of the focus-derived lines reacted by complement-fixation with antisera against the MSV–MuLV complex. That the sarcoma genome was present in each line was demonstrated by rescue of infectious MSV by superinfection of the cells with Rauscher MuLV.

TABLE 1. *Host range of focus-forming virus induced from MSV nonproducer cells*

Nonproducer cells from which virus was induced	MSV titer (FFU/ml) on the following cells		
	NIH/3T3	BALB/3T3	NRK
BALB/3T3 (Ki-MSV)	$10^{1.8}$	Neg	$10^{2.2}$
BALB/3T3 (M-MSV)	$10^{1.4}$	Neg	$10^{1.8}$
NRK (Ki-MSV)	Neg	Neg	$10^{1.4}$

At 6 days after exposure of growing nonproducer cells to 200 μg/ml of BrdU for 24 hrs, culture fluids were harvested, passed through 0.45-nm membrane filters (Millipore Corp.), and assayed for focus formation as described (4). Untreated nonproducer cells yielded no foci.

Induction of focus-forming virus by different agents

Various mutagens and chemical carcinogens were tested for their ability to induce focus-forming virus from MSV nonproducer cells. Each agent was used at a dose that resulted in around 10–50% survival of the cells. With the most active compounds tested: IdC, IdU, and BrdU, as many as $10^{3.5}$ FFU/ml were induced (Table 3). The other activating agents listed have on one or more attempts yielded 1–10 FFU/ml. Chemicals including mitomycin C, 8-azaguanine, nitrosoguanadine, ethidium bromide, and 6-thioguanine have so far failed to induce MSV activity. Similarly, exposure of MSV nonproducer cells to ultraviolet light has been ineffective. The present findings compare favorably with those of Lowy *et al.* (2) for activation of viruses that do not form foci from AKR cells by some of the same chemicals.

DISCUSSION

The present report shows that clonal lines of MSV-transformed nonproducer cells of two species, the mouse and rat, can be induced by chemical agents to make transmissible focus-forming virus. The host range and serologic characteristics of the induced MSV appear to be conferred upon it by an endogenous helper leukemia virus of the host cell rather than by the helper virus of the original transforming virus stock. The induced sarcoma virus maintains its original functions; it is able to transform but is unable to replicate in the absence of helper leukemia virus when transmitted to new cultures. The time course of MSV induction from MSV nonproducer BALB/3T3 cells reflects a similar time course to that of activation of MuLV from normal BALB/3T3 (3). This fits well with the observation that after superinfection of nonproducer cells with MuLV, MSV appears with the same kinetics and efficiency (14).

In addition to the MSV activated from nonproducer mouse cells, it has been possible to detect coexistent MuLV in the induced virus stock by means of its ability to form small, poorly defined XC plaques. Whether there is a rat helper virus in addition to the rat-tropic MSV induced from MSV nonproducer NRK cells has not been directly established, since conditions that allow the virus to persist have not yet been found. Recently, Klement *et al.* (15) have independently shown that MSV can be activated from MSV-transformed NRK cells that do not produce virus. They found that the envelope properties of the induced MSV were unlike those of the mouse virus used to transform the cells. In view of direct evidence that mouse cells contain the genetic information for helper virus production (2, 3) and from the results presented here, it seems quite likely that virus-negative rat cells, too, contain the genetic information for helper virus production. Activation of the sarcoma genome from MSV nonproducer cells probably occurs secondarily to induction of this endogenous helper virus. Whether the MSV and helper leukemia viral genomes coexist at the same or widely separate sites in nonproducer cells requires further study.

The mechanism by which agents such as BrdU induce RNA tumor viruses from virus-negative cells remains to be clarified. Lowy *et al.* have shown that induction of virus by these agents appears to require their incorporation into DNA (2). In the present study, several other chemicals were found to induce MSV from nonproducer cells but at much lower titers than either IdC, BrdU, or IdU. The inability of a number of strongly mutagenic agents such as mitomycin and nitrosoguanidine to activate MSV may imply a more specific effect of the inducers and would appear to rule out the possibility that induction simply results from nonspecific cell toxicity. MSV-transformed nonproducer cells provide an excellent system for examining the process of induction and for the rapid screening of chemical carcinogens and other compounds for their ability to induce RNA tumor viruses from virus-negative cells

TABLE 2. *Properties of focus-derived lines produced by sarcoma virus induced from nonproducer BALB/3T3 cells transformed by Ki-MSV*

Focus-derived cell lines (No. examined)	MuLV CF antigen	Sarcoma virus released*	Supernatant reverse transcriptase	Sarcoma virus yield after superinfection with MuLV†
9	0/9	0/9	0/9	9/9

* Filtered (0.45 nm) culture fluids from each cell line were assayed for focus production on NRK, NIH/3T3, and BALB/3T3 cells pretreated with DEAE-dextran. Negative means no foci in at least five Petri dishes exposed to undiluted culture fluids.

† 7–10 days after infection with 10^6 PFU of Rauscher MuLV, filtered culture fluids were assayed for focus production on BALB/3T3 or NRK cells.

TABLE 3. *Induction of MSV from nonproducer cells by chemicals*

Chemical	Dose (μg/ml)	Induced MSV titer (FFU/ml)
Iododeoxycytidine	200	3.6×10^3
Iododeoxyuridine	200	2.9×10^3
Bromodeoxyuridine	200	1.7×10^3
5-Fluorouracil	0.3	10×10^0
2-Amino-6-mercaptopurine	6.0	2×10^0
Cytocholasin B	12	1×10^0

Growing cultures of nonproducer BALB/3T3 (Ki-MSV) cells were treated for 24 hr with each chemical, then washed twice, and fresh medium was added. At 7 days, culture fluids were passed through 0.45-nm filters and assayed for focus formation on NRK and NIH/3T3 cells pretreated with DEAE-dextran.

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