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Endogenous C-Type Viruses of BALB/c Cells: Frequencies of Spontaneous and Chemical Induction

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A sensitive biological assay has been developed for studying the frequency of virus induction from mouse cells containing poorly infectious endogenous C-type viruses. A low spontaneous frequency of virus activation of one but not a second endogenous virus of BALB/c cells has been demonstrated. The resistance of BALB/c cells to superinfection by the more inducible virus appears to be absolute, whereas resistance to the other virus is only relative. Thus, the evidence indicates that the BALB/c cell exerts differential effects at two levels of regulation on the expression of its biologically distinguishable endogenous C-type viruses.

RNA C-type viruses exist in an unexpressed form in all cells of at least some mouse strains (1, 12). Recent genetic (5, 16, 17) and biochemical (9) evidence indicates that the information for multiple viral copies is integrated within the DNA of the cell. In cells of the BALB/c strain, two biologically distinguishable endogenous viruses are inducible, but neither virus replicates efficiently after activation (5, 16). To study the regulation of their expression, a sensitive biological assay has been developed. This assay involves induction of murine sarcoma virus (MSV) from cells nonproductively transformed by MSV (4, 7). The biological assay for sarcoma virus transformation requires only a single cycle of infection (3, 4), whereas assays for helper leukemia virus generally require many cycles of virus replication. Thus, sarcoma virus, induced in the envelope of the endogenous C-type viruses of the cells (1, 11), provides an indirect but very sensitive indicator for induction of inefficiently replicating endogenous viruses. Using these techniques, it has been possible to detect a very low frequency of spontaneous activation of C-type virus from BALB/c cells. The findings indicate that the spontaneous expression of distinguishable BALB/c endogenous viruses is differentially affected by at least two distinct cellular controls.

MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco modified Eagle medium containing 10% calf serum (Colorado Serum Co., Denver, Colo.) in 50-mm plastic petri dishes (Falcon Plastics). The cells used included clonal lines of continuous, contact-inhibited mouse cells, BALB/3T3 (6) and NIH/3T3 (10), and a line of normal rat kidney (NRK) cells (8). The derivation of Kirsten (Ki) and Moloney (M) MSV-transformed nonproducer BALB/3T3 (K- or M-BALB/3T3) and NRK (K- or M-NRK) lines has been reported (4, 7).

Viruses. Induced BALB viruses 1 and 2 were obtained as described previously (5). Each was propagated in a line of Ki-MSV-transformed nonproducer NRK cells.

Virus assays. Focus formation by MSV was assayed by published methods (7).

Infectious center formation by MSV-activated cells. The conditions for activation of virus from BALB/c cells with iododeoxyuridine (IdU) have been described in detail (1, 4). Unless otherwise stated, immediately after exposure to an inducing agent, cells were treated with mitomycin C (Sigma Chemical Co.) at a concentration of 25 μg/ml for 60 min. This completely inhibited colony formation by K-BALB/3T3 cells without adversely affecting infectious center formation by virus-activated cells. Cultures were washed twice, and 1 h later were treated with 0.1% trypsin in phosphate-buffered saline and transferred to petri dishes containing 10^5 of the appropriate assay cells. These had been plated 24 h earlier in medium containing 2 μg of polybrene per ml (20). Infectious centers of MSV-transformed foci due to virus released from induced cells were scored at 7 to 9 days. Since plating efficiency varied with experimental conditions, chemically induced cells were also transferred to empty petri dishes for a cell count at 24 h. The frequency of virus activation was expressed as the number of MSV infectious centers divided by the total cells adhering to the petri dish at 24 h after transfer.

RESULTS

Infectious center formation by chemically activated K-BALB/3T3 cells. BALB/c cells contain biologically distinguishable endogenous viruses. BALB virus-1 replicates preferentially in National Institutes of Health (NIH) Swiss mouse cells as compared to BALB/c mouse cells (2, 16); BALB virus-2 is not detectably infectious for either NIH Swiss or BALB/c cells but transmits to NRK cells (5). A test was made of the sensitivities of the different assay cells to infectious center formation by MSV activated from a Ki-MSV-transformed nonproducer BALB/c clonal line, K-BALB/3T3. As shown in Table 1, NRK was somewhat more sensitive than NIH/3T3 to infectious center formation by virus-induced cells. Both lines were at least 100-fold more sensitive than BALB/3T3. These results confirmed the previously reported nonpermissiveness of BALB/3T3 cells to superinfection by its endogenous viruses (2, 5, 16) and indicated that NRK was the most sensitive for detecting MSV-activation of K-BALB/3T3.

Kinetics of Ki-MSV-induction by infectious center assay. Previous studies have demonstrated that chemical induction of BALB/3T3 or K-BALB/3T3 cells can result in a transient burst of virus production (1). The time course of Ki-MSV activation in response to IdU was studied by the infectious center technique. After chemical exposure, cultures were washed twice and then transferred at varied intervals for infectious center assay. A 24-h harvest of virus in tissue culture fluids was also assayed at each point. As shown in Fig. 1, around one-tenth of the cells registered as virus-induced over the first 5 days after chemical treatment, despite the fact that, by tissue culture fluid assay, virus was released in a clear peak at 3 days. These results demonstrate the range of sensitivity of the infectious center technique for detection of virus induction. The same fraction of cells registered as induced despite a 20-fold reduction in virus release between days 3 and 5 (Fig. 1).

TABLE 1. *Susceptibility of cell lines to infectious center formation by chemically induced K-BALB/3T3*

Assay cell	MSV infectious centers/total cells[a]
NRK	2.6×10^{-2}
NIH/3T3	1.2×10^{-2}
BALB/3T3	1.0×10^{-4}

[a] Growing K-BALB/3T3 cells were exposed to 10 μg of IdU per ml for 18 h. The cells were washed twice, exposed to mitomycin C at 25 μg/ml for 60 min, and washed twice more. One hour later, they were transferred at 10-fold dilutions in duplicate to petri dishes containing 10^5 assay cells. Focus formation was scored at 7 to 9 days. The total cells were those adhering to a petri dish 24 h after plating as determined by count with a hemocytometer. The results are the average of two experiments.

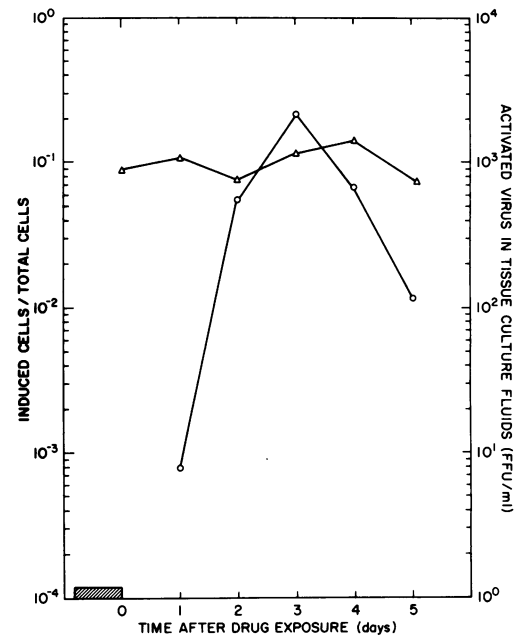

FIG. 1. *Kinetics of IdU induction of K-BALB/3T3 by infectious center assay. K-BALB/3T3 cultures containing 5 × 10^5 cells were exposed to 30 μg of IdU per ml for 20 h at 37 C and then washed twice to remove drug. At subsequent time intervals, the cells were transferred for infectious center assay on NRK cells as described in Materials and Methods. At each time point, a 24-h harvest of tissue culture fluids was assayed for focus-forming virus on NRK cells. The virus released per 10^6 cells was determined by cell count at each time point. The results are the average of two experiments. Symbols: Δ, induced cells/total cells; O, activated virus in tissue culture fluids.*

Dose response for IdU induction by infectious center assay. The effect of IdU concentration on the fraction of virus-activated cells was tested next. As shown in Fig. 2, exposure of K-BALB/3T3 cells to increasing drug doses above a threshold level of 0.1 μg/ml resulted in an increasing fraction of virus-induced cells. The response reached a plateau with IdU treatment above 10 μg/ml. In general, the amount of activated sarcoma virus in culture fluids at the peak of virus release was proportional to the number of induced cells detected by infectious center assay. However, at high drug levels there was a clear decrease in the amount of virus released per activated cell (Fig. 2). Since IdU

treatment at these doses markedly impaired cell viability, as evidenced by visible signs of cell toxicity and decreased colony formation, the results suggest that the ability of an induced cell to register as an infectious center was more resistant to IdU toxicity than was its ability to release maximal amounts of virus.

Spontaneous virus leakage from BALB/c cells. The frequency of spontaneous virus activation has been quantitated with mouse cells that contain highly infectious endogenous viruses (14). Because of the poorly infectious nature of its endogenous viruses, the frequency of spontaneous virus activation from BALB/c cells has been more difficult to measure. The availability of MSV nonproducer transformants of this strain made it possible to study spontaneous virus activation frequency of these cells. Exponential-phase cultures of different MSV nonproducer clones and subclones were assayed for virus activation in the absence of treatment with IdU. As shown in Table 2, the frequency of spontaneous virus-induced cells ranged from 1.1 $\times 10^{-6}$ to 4.5 $\times 10^{-6}$ with the different clones tested. The frequency of virus-activated cells in subclones tested many generations later was similar. These findings indicate that virus activated at any given time did not spread to other cells.

TABLE 2. *Spontaneous virus activation from BALB/c-derived MSV-transformed nonproducer clones*

Cell line	MSV-infectious centers/total cells[a]
K-BALB/3T3	
Clone 1	4.5×10^{-6}
Subclone 1	2.8×10^{-6}
Subclone 2	5.1×10^{-6}
K-BALB/3T3	
Clone 2	3.2×10^{-6}
Subclone 1	7.3×10^{-6}
M-BALB/3T3 clone 1	1.1×10^{-6}

[a] Growing cultures of each cell line were exposed to mitomycin C (25 μg/ml $\times$ 1 h) and then transferred at 10-fold cell dilutions in duplicate to petri dishes containing 10^5 NRK cells for infectious center assay. K-BALB/3T3 Cl 1 and Cl 2 are clonal nonproducer lines derived from diffferent foci of Ki-MSV-transformed BALB/3T3 cells (7). M-BALB/3T3 clone 1 is a nonproducer clone of M-MSV-transformed BALB/3T3 cells (4). The results are the average of two experiments with each line.

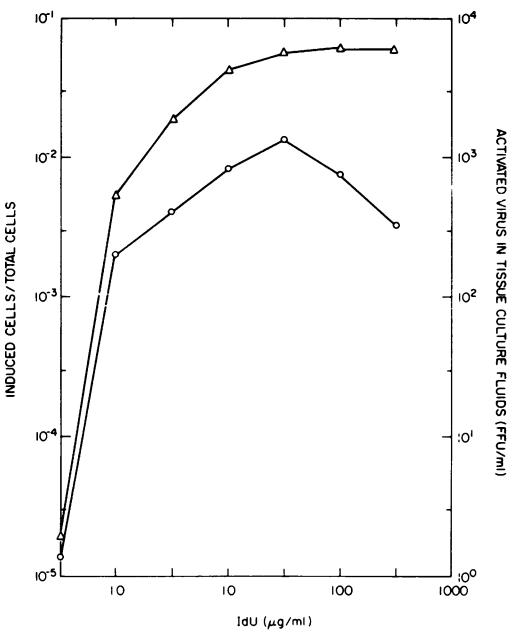

FIG. 2. *Dose response for IdU-induction by infectious center assay. K-BALB/3T3 cultures containing 5 $\times$ 10^5 cells were exposed to varying IdU concentrations for 20 h. After two washes with medium, the cells were transferred for infectious center assay on NRK cells as described in Materials and Methods. Parallel cultures were tested for focus-forming virus in tissue culture fluids at the peak of virus activation (3 days) on NRK cells. The results are the average of two experiments. Symbols: Δ, induced cells/total cells; O, activated virus in tissue culture fluids.*

The effect of the growth state of the cells on spontaneous virus activation from one K-BALB/3T3 clone was studied. Stationary phase cells were subcultured and tested at different time intervals for the frequency of virus induction. At each point, tissue culture fluids of those cells were assayed for the presence of sarcoma virus. As shown in Fig. 3, exponentially growing cells had an almost threefold higher frequency of virus activation than confluent cultures. Spontaneously activated virus was also detected at very low levels in tissue culture fluids and was found to vary with the growth state of the cells. While confluent K-BALB/3T3 cultures released no detectable virus (less than 0.05 focus-forming units [FFU] per ml per 10^6 cells), the same cells, actively growing 3 days later, released 0.65 FFU per ml per 10^6 cells.

Host range of spontaneously induced virus. The ability of two BALB/c endogenous viruses to persist after chemical induction has been shown to be affected differently by at least one host cell regulatory factor (5, 16). Thus, it was reasoned that there might also be differences in cellular control over the spontaneous activation of the two viruses. To study this question, the host range pattern for infectious center forma-

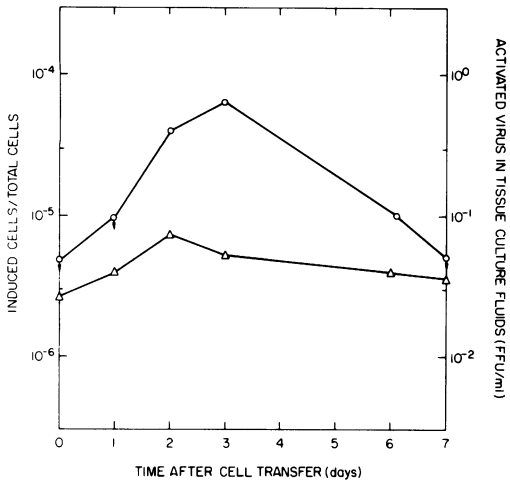

FIG. 3. *Effect of cell growth on spontaneous C-type virus induction. Confluent cultures of K-BALB/3T3 (3 days after the last medium change) were transferred at 5 × 10⁵ cells per plate (day 0) and assayed on NRK cells at various times thereafter for infectious center formation and for focus-forming virus in tissue culture fluids. The sarcoma virus in tissue culture fluids per 10⁶ cells was determined by cell count at each time point. The values at 0 and 7 days reflect those for confluent cultures. The results are the average of three experiments. Symbols: Δ, induced cells/total cells; O, activated virus in tissue culture fluids.*

tion by spontaneously activated K-BALB/3T3 cells was compared with the pattern of infectious center production by cells chronically replicating Ki-MSV with either the BALB: virus-1 or BALB: virus-2 pseudotype. As shown in Table 3, the efficiency with which BALB: virus-1-producing cells formed infectious centers was very similar on NIH/3T3 and NRK cells. Cells producing sarcoma virus with this pseudotype also registered on BALB/3T3 cells, but with 12- to 16-fold less efficiency. BALB: virus-2-producing cells formed infectious centers at high efficiency on NRK, but there was no evidence of infection of either mouse line. Thus, the mouse cells appeared to be absolutely resistant to the transmission of Ki-MSV with BALB: virus-2 envelope properties. The pattern of infectious center formation by spontaneously induced K-BALB/3T3 closely resembled that of cells producing BALB: virus-2; focus-formation was detected at low frequency and only on NRK. From the number of foci scored on NRK, it would have been possible to detect a 50-fold lower frequency on either NIH/3T3 or BALB/3T3 (Table 3). Spontaneously activated virus was also detected in tissue culture fluids and was infectious only for NRK. These findings indicate that the virus spontaneously activated from K-BALB/3T3 cells was predominantly BALB: virus-2.

DISCUSSION

The present studies show that the BALB/c mouse cell exerts controls over the spontaneous expression of its two endogenous viruses at distinct levels affecting virus activation and persistence. At the level of spontaneous activation, BALB: virus-1 was found to be much more tightly restricted than BALB: virus-2. At the level of virus persistence, however, BALB/c cells were shown to be absolutely resistant to exogenous infection by BALB: virus-2, but only relatively resistant to BALB: virus-1. The latter of the two regulatory levels appears to be the more effective in preventing spontaneous virus expression both in vitro and in vivo. Thus, in instances where spontaneous virus production has been observed with BALB/c cells in tissue culture, the virus has closely resembled BALB: virus-1 (2, 15, 19). Similarly, in vivo, an increasing percentage of BALB/c mice become virus positive with age (13). Virus isolated from such animals has properties indistinguishable from those of BALB: virus-1. Despite the apparent inability of BALB: virus-2 to persist in vivo, evidence has recently been obtained that it is spontaneously activated. Neutralizing antibodies to it have been detected in the sera of BALB/c mice (unpublished observations).

The frequency of spontaneous virus activation from BALB/c cells in the present studies is at least 100-fold higher than the reported frequency of spontaneous virus activation from

TABLE 3. *Host range pattern of infectious center formation by spontaneously activated BALB/c cells*

Cells tested	Ratio of virus-releasing cells registering on[a]	
	NIH/3T3: NRK	BALB/3T3: NRK
Chronically replicating MSV-pseudotype of:		
BALB:virus-1	1.3×10^{0}	8×10^{-2}
BALB:virus-2	$<1 \times 10^{-4}$	$<1 \times 10^{-4}$
K-BALB/3T3	$<2 \times 10^{-2}$	$<2 \times 10^{-2}$

[a] Cell lines chronically replicating BALB:virus-1 or -2 pseudotypes of Ki-MSV were obtained by superinfection of K-NRK nonproducer cells with either helper virus. Infectious center assay of virus-producing lines was performed as in the standard assay with the addition of two rinses with 1% trypsin for 1 to 2 min per rinse prior to cell transfer. This treatment destroyed infectivity of more than 99% of free or absorbed virus. The results are the average of two experiments.

clonal lines of AKR embryo cells (14). It should be noted that induction was measured with BALB/c cells nonproductively transformed by MSV and utilized sarcoma virus as a sensitive indicator of the cell's endogenous helper viruses. In contrast, activation of AKR cells was measured by an assay for leukemia virus, the XC plaque method (14). Thus, the differences in spontaneous virus activation frequencies with BALB/c and AKR cells may reflect differences in sensitivities of the assays and/or differences in the cells themselves.

The biological assay of sarcoma virus activation from MSV nonproducer cells (1, 5, 11) has been shown to be more sensitive under certain conditions than any available biochemical method for detection of C-type virus induction (18). The infectious center method utilized in the present studies for quantitating sarcoma virus-activated nonproducer cells increases the sensitivity of this assay. Compared to quantitation of activated virus in tissue culture fluids, the infectious center technique was found to be less affected by such factors as cell toxicity and variations in kinetics of virus release. This may prove useful both in testing for new chemical inducers and in attempts to detect inducible C-type viruses of other species.

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