Studies of FBJ Osteosarcoma Virus in Tissue Culture. II. Autoinhibition of Focus Formation [1]

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SUMMARY—A high-titered non-focus-forming virus, FBJ-MuLV (murine leukemia virus), present in FBJ tumor preparations, inhibited significantly the expression and production of FBJ-MuSV (murine sarcoma virus) in tissue culture. This "autoinhibition" was comparable to that observed when a 3- to 4-log excess of infectious MuLV was added to standard MuSV. The degree of inhibition was influenced by the tropism of the MuLV (or the ease of spread and propagation of MuLV in certain cells), multiplicity of infection by MuLV, amount of excess MuLV, and ability of the MuSV-transformed cells to replicate independently. The FBJ MuLV-MuSV complex may be a model system for the detection of sarcoma viruses in spontaneous tumors in various animals where inhibition by excess nontransforming virus could be an important biologic phenomenon.—J Natl Cancer Inst 54: 615–619, 1975.

A MURINE SARCOMA VIRUS (FBJ-MuSV) and a non-focus-forming murine leukemia virus (FBJ-MuLV) have been isolated in tissue culture from osteosarcomas induced by extracts from a passaged bone tumor that occurred spontaneously in a male CF-1 mouse (*1–3*). They are members of the RNA MuLV-MuSV complex; they have the group-specific MuLV antigen, and a density of 1.16g/cm^3, are chloroform sensitive, and by electron microscopy appear as typical C-type particles (*2–5*).

This MuSV is unusual in several respects. In contrast to the most widely studied MuSV strains, it was isolated from a spontaneous tumor rather than from laboratory-passaged virus preparations. Its tissue specificity in vivo is different from that of other MuSV's, since the sarcomas often arise in bone rather than connective tissue. In tissue culture assays, it is remarkable in transforming secondary mouse embryo cells into autonomously replicating transformed cells, as evidenced by one-hit kinetics of focus formation and failure of antiserum added after infection to suppress focus formation (*3*). Also, focus induction by FBJ-MuSV is slower than that by other MuSV strains: The foci are morphologically different; focus formation is markedly affected by the medium used; the titer of the focus-forming component in FBJ-MuSV stocks is lower; and the ratio of MuLV to MuSV is much higher. Understanding these special features of a naturally occurring MuSV may be particularly important in the detection of other sarcoma viruses and evaluation of the possible function of such viruses in the etiology of various mouse sarcomas.

A particularly important technical problem in detection and quantitation of the FBJ-MuSV component is the prozone, or "autoinhibition" effect, seen in tissue culture assays. Here we analyze this phenomenon and compare it with MuLV inhibition of focus formation by other MuSV strains.

MATERIALS AND METHODS

Viruses.—The FBJ-MuSV and -MuLV viruses were obtained as described in (*3*). For most experiments, one preparation of tissue culture-derived virus was used. The Harvey MuSV (H-MuSV) and Moloney MuSV (M-MuSV) were grown in NIH Swiss mouse-embryo (NIH-ME) cells in our laboratory. They were originally obtained from Dr. Jennifer Harvey and Dr. John Moloney, respectively. The Rauscher MuLV (R-MuLV) was provided by Dr. Frank Rauscher and passaged in NIH-ME tissue culture in our laboratory.

Tissue culture and media.—NIH-ME, BALB/3T3, and NRK cells were obtained and cultivated as previously described (*3, 6–8*). The MSV-8 cell line, a non-virus-producing, M-MuSV-transformed BALB/c3T3 line (*9*), was provided by Dr. Stuart Aaronson. A non-virus-producing NRK line transformed by H-MuSV (NRK-Harvey) was derived in our laboratory (*10*). Both cell lines contain a MuSV genome rescuable by MuLV.

Virus detection.—MuLV was detected by the XC plaque assay (*11*). Titers are expressed as plaque-forming units (PFU)/0.4 ml. Focus formation assays for MuSV detection were conducted as described in (*3, 7, 8, 12*). Titers are expressed as focus-forming units (FFU)/0.4 ml.

RESULTS

Inhibition in FBJ Preparations

When FBJ-MuSV was titrated in mouse and rat cells, starting with undiluted virus, a prozone was consistently observed in which focus formation was inhibited. Inhibition occurred when the virus preparation was tested undiluted or diluted up to and including 1:4 (text-fig. 1). However, at about a 1:10 dilution of the FBJ-MuSV preparation, the kinetics of focus formation became one-hit. This prozone in the dose-response curve was not observed in titrations of isolates of other MuSV's containing approximately the same amount of MuLV (10^8 PFU). For those, however, the titer of MuSV was within 1–2 $\log_{10}$ of the MuLV titer, and focus titrations were done with higher dilutions of virus. FBJ preparations have an excess of 3–4 $\log_{10}$ of MuLV (*3*), which suggests that focus formation by high concen-

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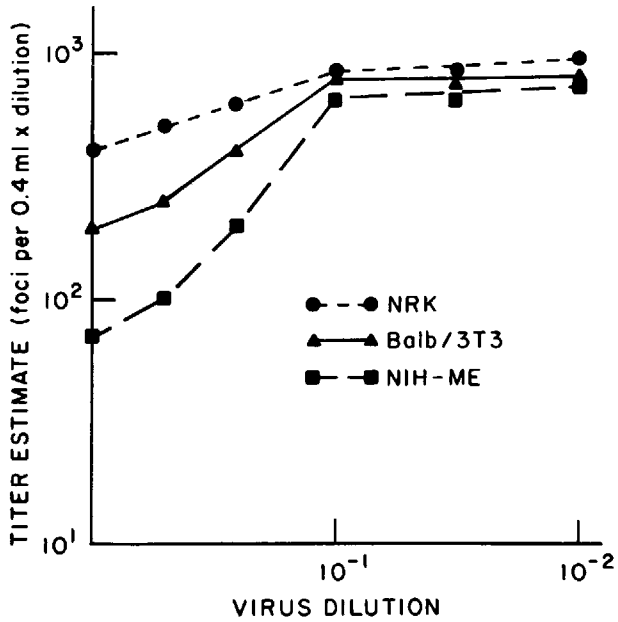


TEXT-FIGURE 1.—Dose-response relations in titrations of an FBJ preparation in NRK, BALB/c, and NIH-ME cells.

trations of FBJ-MuSV is inhibited by this unusual excess of FBJ-MuLV.

Supporting this interpretation is the observation (text-fig. 1) that the prozone was more pronounced in the NIH-ME cells than in the BALB/c and NRK cells, which are relatively nonpermissive to propagation of the FBJ-MuLV, an N-tropic virus (*3*, *13*).

The difference in extent of inhibition in various cell lines was observed also in experiments to determine the effect of additional MuLV on focus-forming titers of FBJ-MuSV. FBJ-MuLV and Moloney MuLV (M-MuLV) were added in undiluted amounts ($10^{6.3}$ and $10^{6.0}$ PFU, respectively) to cultures simultaneously infected with a standard amount of FBJ-MuSV.

No additional inhibition occurred when the undiluted FBJ-MuSV preparation (i.e., $10^{3.2}$ FFU) was tested in the presence of the added MuLV (table 1, A). With lower concentrations of FBJ-MuSV (table 1, B and C), inhibition by added FBJ-MuLV was observed only in NIH-ME cells, and not in BALB/3T3 cells. However, M-MuLV, an NB-tropic virus, when added in excess, effectively suppressed focus formation in both NIH-ME and BALB/3T3 cells simultaneously infected with FBJ-MuSV. FBJ-MuLV and M-MuLV produced comparable inhibition of FBJ-MuSV when tested in sensitive cells (table 1, C).

The effect of autoinhibition in the FBJ-MuSV system as compared to other MuSV systems can be seen from the results of focus assays in which 1,000 FFU (as determined by titration in NIH-ME cells with an optimal amount of helper) of standard MuSV were tested in the presence of the same high titer of FBJ-MuLV found in a standard FBJ preparation ($10^{6.3}$ PFU). The amount of MuSV used was equivalent to that present in an undiluted FBJ-MuSV preparation. Table 1 (D and E) shows that these dilutions of H-MuSV and M-MuSV in the absence of added MuLV gave two to three times as many foci as undiluted FBJ virus containing the same number of MuSV particles. This number of foci was less than the quantity of MuSV particles, since their dilution also resulted in dilution of the accompanying MuLV which is necessary for their focus formation in NIH-ME cells (*7*,*8*,*10*,*14*). When these dilutions were tested with undiluted FBJ-MuLV, focus formation was substantially inhibited.

In general, production of progeny MuSV varied directly with the number and size of the foci in each culture; inhibition of focus formation resulted in proportionally lower titers of MuSV recovered in supernatants: e.g., a 2-$\log_{10}$ decrease in focus formation in those cultures infected with H-MuSV or M-MuSV and excess MuLV gave a 2-$\log_{10}$ drop in progeny production. An absence of foci, however, did not necessarily denote the absence of MuSV progeny.

TABLE 1.—*Effect of added MuLV on MuSV focus formation*

Virus[a]	MOI[b] MuLV	Number of foci/dish	
		NIH-ME	BALB/-3T3
A) FBJ-MuSV (1,600 FFU & $10^{6.3}$ PFU)	6	62	----------
" " " + FBJ-MuLV ($10^{6.3}$ PFU)	11	60	----------
" " " + M-MuLV ($10^{6.0}$ PFU)	9	60	----------
B) FBJ-MuSV (200 FFU & $10^{5.4}$ PFU)	0. 7	----------	125
" " " + FBJ-MuLV ($10^{6.3}$ PFU)	6	----------	125
" " " + M-MuLV ($10^{6.0}$ PFU)	4	----------	20
C) FBJ-MuSV (80 FFU & $10^{5.0}$ PFU)	0. 3	24	----------
" " " + FBJ-MuLV ($10^{6.3}$ PFU)	6	4	----------
" " " + M-MuLV ($10^{6.0}$ PFU)	3	4	----------
D) H-MuSV (800 FFU & $10^{3.4}$ PFU)	0. 01	130	----------
" " " + FBJ-MuLV ($10^{6.3}$ PFU)	6	3	----------
E) M-MuSV (1,600 FFU & $10^{4.5}$ PFU)	0. 1	180	----------
" " " + FBJ-MuLV ($10^{6.3}$ PFU)	6	3	----------

[a] MuSV titers determined in NIH-ME cells with optimal dose of MuLV helper. Concentrations of MuSV and MuLV expressed as FFU and PFU, respectively.

[b] Multiplicity of infection (PFU/cell).

Some cultures, in which focus formation was inhibited by high titers of FBJ-MuLV, produced good titers of progeny FBJ-MuSV (e.g., $10^{2.6}$ FFU). In testing for focus-forming activity in supernatants from cultures receiving high-titered MuLV, dilutions had to be made to avoid the inhibitory effect of the excess progeny MuLV.

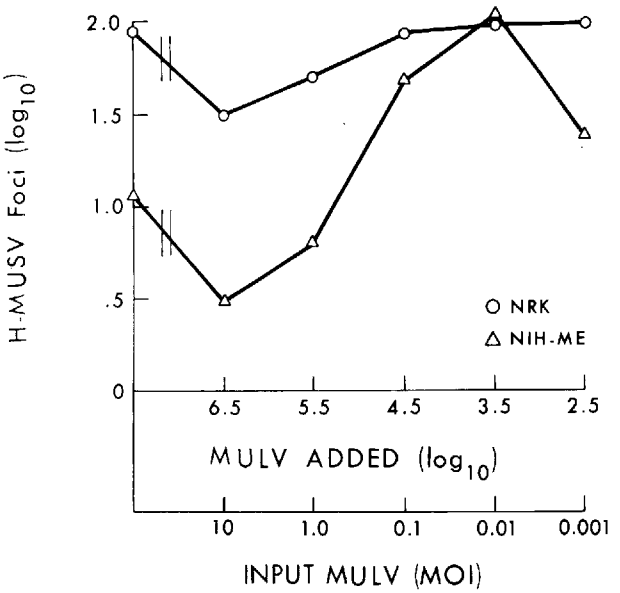
In attempts to diminish FBJ-MuLV excess, the FBJ-MuSV preparation was passed through less permissive cells such as NRK and BALB/3T3 cells. Although FBJ-MuLV titers decreased, so did the FBJ-MuSV titers; on subsequent passage in NIH-ME cells, the FBJ-MuLV titer again reached 10^{6-7} PFU and was 3–4 $\log_{10}$ higher than that of FBJ-MuSV.

Effects of Various Proportions of MuLV:MuSV on Degree of Inhibition of H-MuSV Foci

The effect of the ratio of MuLV to MuSV on focus formation by a standard MuSV was tested by the inoculation of a single dilution of H-MuSV onto mouse and rat cell monolayers (350,000 cells) simultaneously infected with various concentrations of MuLV. In the mouse cells, MuLV was required for focus formation, since the MuSV-infected cells cannot replicate independently (*8*, *10*, *14*); for this reason, both a helper and inhibitory effect of MuLV on focus formation could be demonstrated. In the rat cells, on the other hand, only the inhibitory effect of MuLV was observed, since MuSV-infected rat cells themselves can propagate independently to form a focus (*10*). The Harvey strain was selected because it grows equally well in both mouse and rat cells. At the dilution used ($10^{2.0}$ FFU), the MOI of H-MuSV was about 0.0003 FFU/cell and the MOI of the accompanying H-MuLV was about 0.001 PFU/cell. M-MuLV was added to the NIH-ME cells; R-MuLV was added to NRK cells, since this virus grows better in rat cells than does M-MuLV.

Text-figure 2 illustrates the balance between inhibition and enhancement of MuSV focus formation by MuLV in NIH-ME cells. An MOI of 1–10 infectious MuLV PFU/cell, or 3–4 logs more M-MuLV than H-MuSV, inhibited focus formation in NIH-ME cells significantly. On the other hand, lower MOI's (0.1–0.01 PFU/cell), representing an excess of MuLV of 1–2 logs, enhanced focus formation. When the MOI of MuLV added was approximately equal to the amount of H-MuLV present (0.001 PFU/cell), focus formation increased but slightly as compared to that of control plates infected by the H-MuSV alone. The difference between dilutions of helper virus giving marked inhibition or optimal enhancement by MuLV in the NIH-ME cells was therefore only about 100-fold.

In rat cells, inhibition of H-MuSV focus formation was also observed with high MOI's of MuLV. The degree of this inhibition, however, was less than that noted in the mouse cells. Unlike in the mouse cell system as described above, no enhancement of H-MuSV titer was observed in the rat cells with higher dilutions of MuLV, since focus formation in these cells does not require MuLV (*8*, *10*).



TEXT-FIGURE 2.—Effect of MuLV added to cultures receiving H-MuSV. NRK and NIH-ME cells (350,000/plate) inoculated simultaneously with 100 FFU of H-MuSV and various concentrations of R-MuLV and M-MuLV. Focus-forming titers of H-MuSV were determined in duplicate plates. Quantity of MuLV added is given both as absolute titer ($\log_{10}$) expressed in PFU/0.4 ml and as input MOI.

Focus Formation in Cells Chronically Infected With MuLV

Undiluted FBJ-MuSV and M-MuSV preparations were inoculated onto mouse (NIH-ME and BALB/c) and rat cells chronically infected with either FBJ-MuLV or M-MuLV. No focus formation or progeny production by either MuSV strain was noted in these cultures.

Effect of MuLV Added to MuSV-Transformed Cells

To test the possibility that inhibition of focus formation might result from a reversion of MuSV-transformed cells back to normal cells on superinfection by excess MuLV, non-virus-producing transformed cell lines were used. Nonproducing BALB/3T3 cells (MSV-8 line) (*9*) and nonproducing rat cells (NRK-Harvey line) (*10*) were superinfected with M-MuLV and R-MuLV, respectively, at inputs from 0.01 to 100 PFU/cell. The cells, observed for over 4 weeks, maintained their morphologic alteration, continued to multiply at the same rate, and began releasing infectious MuSV and MuLV by the 1st week after inoculation with MuLV.

DISCUSSION

The data show that FBJ-MuSV preparations produce a significant degree of autoinhibition of focus formation when tested at low dilutions. This inhibition is probably not caused by interferon, since RNA tumor virus infection does not induce detectable amounts of interferon (*15*, *16*). Moreover, an inhibitor such as that found in cultures of NZB cells does not appear to be responsible, since this factor is equally effective in both N- and B-type mouse cells and is

usually demonstrable only after concentration of the culture supernatants (*17, 18*). Rather, the inhibition is adequately explained by the large excess of MuLV present in FBJ-MuSV preparations.

Inhibition of focus formation by a sarcoma virus by preinfection with a related nontransforming virus was first reported by Rubin in 1960 (*19*). Subsequently, Steck and Rubin (*20, 21*) showed that simultaneous infection with a high multiplicity of avian leukosis virus also inhibits focus formation by a sarcoma virus. Although it is convenient to consider the resistance produced by coinfection and preinfection as separate phenomena ("early" and "late" inhibition), they probably involve the same mechanism of resistance: i.e., viral antigens in or on the cell membrane interfering with the penetration-uncoating stage of the infection (*20, 22*). The late type of inhibition is well known in the murine system (*23*), whereas the early type has received little attention.

To understand the dynamics of autoinhibition caused by the MuLV present in FBJ-MuSV preparations, it is important to recognize two mechanisms for focus formation by MuSV's. In one, proliferation of the MuSV-infected cell results in a focus; in the other, cells do not proliferate, and foci form only after infection of nearby cells by progeny MuSV (*7–10, 14*). This second system depends on adequate MuLV for focus formation, because replication of the MuSV requires MuLV. Inhibition of focus formation therefore can take place as an early event in which initial infection by MuSV is blocked by MuLV, or as a late event where infection of neighboring cells by progeny MuSV is prevented by the prior infection of these cells by MuLV.

With cells in which the MuSV-infected cell can propagate independently into a focus, only the early inhibition is important. Since FBJ-MuSV focus formation occurs by this mechanism, the prozones in the titration curves reflect this early inhibition. In the prozone region of the dose-response curve (text-fig. 1), FBJ-MuSV focus formation was insensitive to further inhibition by excess MuLV (table 1, A). Since the MuLV present in that FBJ preparation was already 10^6 PFU/ml, an increase in the input of MuLV twofold was not sufficient to interfere further with the input MuSV. When the FBJ preparation was diluted, which decreased the input of FBJ-MuLV, one-hit kinetics were observed and focus induction was then sensitive to inhibition by excess MuLV (table 1, B and C). Early inhibition was also observed in rat cells when MuLV was added at an MOI of 1–10 PFU/cell to a fixed dilution of H-MuSV (text-fig. 2). In those systems where the transformed cells can replicate (FBJ-MuSV in NIH-ME and H-MuSV in NRK cells), early inhibition of focus formation requires a relatively high MOI of MuLV infection (1–10 PFU/cell). A similar conclusion has been made about the number of infectious virus particles/cell needed to interfere with Rous sarcoma virus (RSV) focus formation in chick cells (*20*); RSV-transformed chicken cells also can multiply independently.

On the other hand, early-passage mouse embryo cells infected by other strains of MuSV cannot replicate, and a focus is formed only by infection of neighboring mouse embryo cells by progeny MuSV (*8, 10, 14*). In this type of cell system, both early and late inhibitory events can occur. Late inhibition occurs when cells surrounding a cell containing MuSV are infected by MuLV before infection with progeny MuSV has taken place. This prevents the subsequent spread of MuSV progeny and the formation of recognizable MuSV foci. This late type of inhibition was responsible for the complete suppression of MuSV focus formation and progeny production observed when cells chronically infected with FBJ-MuLV or M-MuLV were challenged with FBJ-MuSV and M-MuSV. The presence of both early and late inhibitory events explains the greater magnitude of MuLV inhibition of foci formed by M-MuSV and H-MuSV in NIH-ME cells (table 1, D and E) than those formed by FBJ-MuSV. It also suggests why a difference in MuLV input of only 2 $\log_{10}$ can determine whether MuLV "enhances" or "inhibits" focus formation in NIH-ME cells. The optimal enhancing amount of helper MuLV represents an MOI of 0.01 to 0.1 PFU/cell; this amount presumably provides the optimum balance between helping MuSV replication by coinfection and superinfection of a previously MuSV-infected cell (*10, 14*) and leaving enough cells uninfected that second-cycle MuSV infection is not blocked. However, an input of 1–10 infectious MuLV particles/cell produces inhibition by both the early and late mechanisms.

The studies presented here illustrate the complex of factors that affect detection and quantitation of MuSV genomes. The effect of autoinhibition is particularly important when one considers that if the FBJ-MuSV-infected cell did not have the unusual ability to replicate as a colony of transformed cells, or if the excess of MuLV had been even higher, focus formation by this virus might not have been detected. Similarly, previous data have suggested that certain MuLV preparations may contain small amounts of MuSV which cannot be detected by standard in vitro techniques because of the great excess of MuLV (*24*). Whether this autoinhibition can also produce false negative results in in vivo assays remains to be determined.

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