

## Titration of Murine Leukemia Viruses with Rat Cell Line RFL

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Normal rat embryo cells (RFL) form syncytia after infection with murine leukemia virus. The assay for counting the number of syncytium foci produced in RFL cells is a sensitive method for a direct infectivity assay of murine leukemia virus.

Concentrated murine leukemia virus (MuLV) or MuLV-producing cells induce XC cell fusion within an hour, leading to syncytium formation (1, 2, 6). Since the fusion of XC cells (10) required high concentrations of virions, the XC cells could not be used for the direct assay of type C RNA viruses.

I have previously shown that normal rat fibroblast cells (RFL) in culture responded to MuLV by syncytium formation between days 7 and 10 after infection (3, 4). The present paper describes the development of a direct assay of MuLV utilizing the syncytium formation.

RFL cells, an established cell line derived from cultures of WKA rat embryo lung, were obtained from R. Takaki, School of Medical Science, Kyushu University (R. Takaki, unpublished data), at approximately the 30th passage level, and had been cultured in our laboratory. NIH-3T3 cells were obtained from H. Yoshikura, Institute of Medical Science, Tokyo University. XC cells were acquired from R. Mori, Department of Virology, Kyushu University, School of Medicine. SC-1 cells were obtained from V. Klement, University of Southern California. Cells were cultivated in Eagle minimum essential medium (Nissui, Japan) with 10% heat-inactivated (at 56°C for 30 min) calf serum (Chiba Serum Research Institute, Japan) in an incubator with humidified air containing 5% CO<sub>2</sub>. Moloney-MuLV was obtained from N. Ida, Toyo Kogyo Hospital, Hiroshima. Culture medium from virus-infected NIH-3T3 cells was used after filtration through a 0.45- $\mu$ m filter. Wild mouse virus 1504E, mouse tropic clone (titer in SC-1, 10<sup>5.16</sup>), was obtained from V. Klement, University of Southern California.

The XC test was carried out by the method of Rowe et al. (9) with the NIH-3T3 cells or SC-1 cells.

RFL cells ( $3 \times 10^5$  cells) were plated onto a 6-

cm glass petri dish and cultured at 37°C in Eagle minimum essential medium with 10% calf serum. One day after plating, the medium was removed, and sparse monolayers were infected with 0.1 or 0.2 ml of diluted viruses. After adsorption at 37°C for 1 h, each plate was supplemented with 5 ml of medium and incubated at 37°C in 5% CO<sub>2</sub>. On day 3 after infection, the plates were supplemented with 4 ml of additional medium. On day 6, cultures were fixed with 100% methanol and stained with 5% Giemsa, and syncytia with four or more nuclei were counted under a microscope at  $\times 40$  magnification in, usually, 40 fields (160 mm<sup>2</sup>). The number was corrected by subtracting the average number in parallel uninfected cultures. The number of syncytia with four or more nuclei in uninfected cultures was usually zero on day 2 and less than four on day 9 after plating in 40 fields (160 mm<sup>2</sup>). Syncytium focus (2 to 10 syncytia in close proximity) was never observed in the uninfected cultures.

RFL cells in culture on a glass substratum were fusiform in shape and measured approximately 35  $\mu$ m in diameter. They exhibited the typical density-dependent inhibition of cell division. The first appearance of syncytia was observed at day 2 after infection, and syncytium foci (2 to 10 syncytia in close proximity) were observed on day 5 (Fig. 1). When such a focus was counted as one positive cell, the number of syncytia increased with time and reached a plateau on day 6 to 7 after infection. The number of syncytium foci was proportional to the dilution, as shown in Table 1. The size of syncytium foci formed in RFL cultures was small as compared to those in XC assay. The syncytium-forming units (SFU) of virus samples could be calculated, and it was comparable to the PFU obtained by a mouse cell-XC test. The SFU-PFU ratio ranged between 1:1 and 4:1 with Moloney-MuLV and wild mouse virus

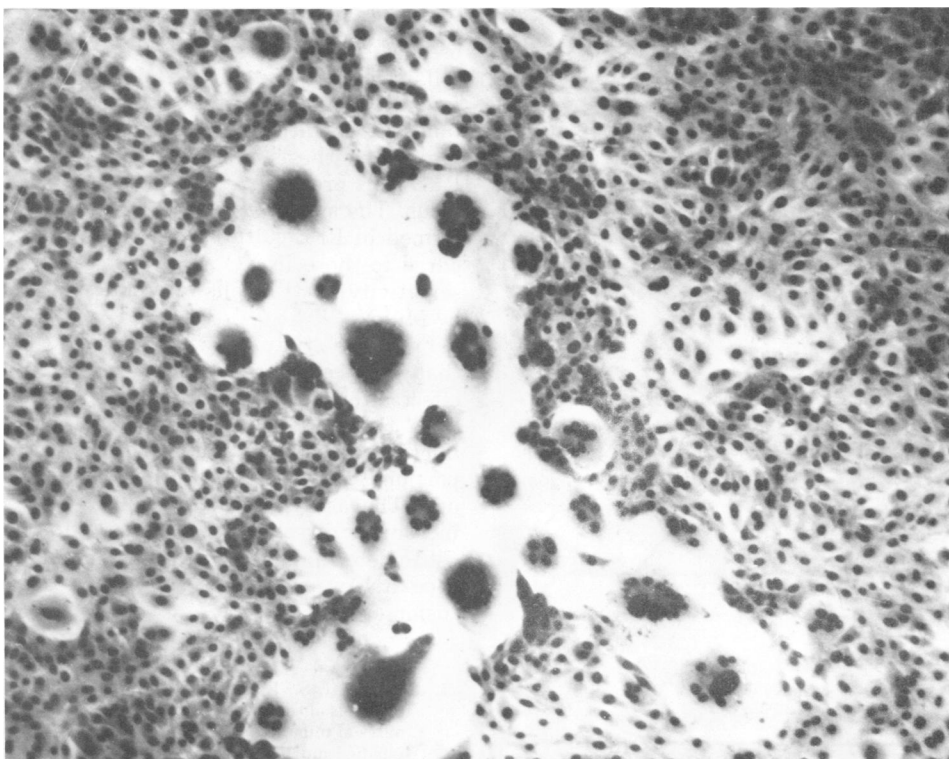


FIG. 1. Giemsa-stained syncytium focus induced by Moloney-MuLV on RFL cells 6 days after infection.  $\times 160$ .

1504E, respectively. However, a definitive comparison of the two methods may not be made at this moment, since DEAE-dextran, known to improve the efficiency of infection, was not used in the XC assay in this experiment. To exclude the disturbance by "fusion from without," DEAE-dextran was not used in this experiment, though DEAE-dextran also improved the efficiency of syncytium formation.

Syncytium formation did not occur when virus was treated prior to infection with specific antiserum against purified virions, and it also did not occur when virus was filtrated through a membrane filter of 0.05- $\mu$ m pore size (Sartorius, West Germany) prior to infection. Such formation was also lost after heating at 56°C for 15 min, or when treated by ether.

The effect of UV irradiation of MuLV on syncytium formation was studied. Moloney-MuLV was irradiated with UV light for various periods of time. As shown in Fig. 2, the number of plaques decreased proportionally to the length of irradiation time. Similar UV-inactivation curves were obtained when the same samples were assayed by the XC test.

Temporary inhibition of DNA synthesis immediately after infection drastically reduced

TABLE 1. Comparative plaque titration of Moloney-MuLV with RFL cells and XC cells

Dilution of virus	No. of plaques/plate <sup>a</sup>	
	RFL	XC
3 <sup>-4</sup>	Confluent	129, 135
3 <sup>-5</sup>	137, 120	37, 68
3 <sup>-6</sup>	42, 48	9, 10
3 <sup>-7</sup>	19, 7	3, 3

<sup>a</sup> Experiments performed in duplicate.

the number of syncytia counted at day 6. After virus adsorption, cultures were incubated in growth medium containing 10  $\mu$ g of cytosine arabinoside per ml for 3 h at 37°C, washed twice with phosphate-buffered saline, and incubated in growth medium for syncytium formation. Treatment with cytosine arabinoside under the conditions described inhibited incorporation of [<sup>3</sup>H]thymidine (5 Ci/mmol, 0.1  $\mu$ Ci/3 ml of culture medium) into acid-insoluble materials during the 3-h period to 5%. The treatment with cytosine arabinoside from 0 to 3 h after virus adsorption inhibited almost completely the formation of syncytium, whereas the same treatment from 8 to 11 h had no effect at all.

The behavior of RFL cells after infection of

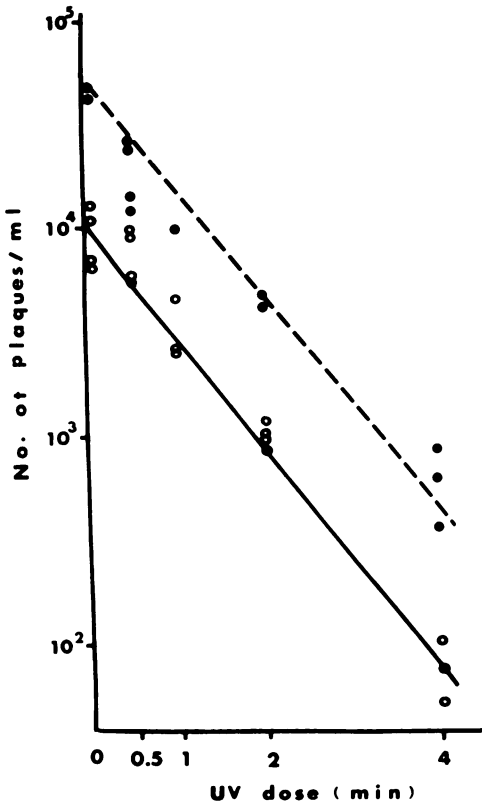


FIG. 2. Effect of UV irradiation of Moloney-MuLV on syncytium foci formation. One milliliter of virus in 6-cm glass petri dishes was irradiated by UV light at a distance of 20 cm from a germicidal mercury lamp (GR-1010B, 14 W; Toshiba, Japan) for different periods of time, and surviving virus was titrated with XC cells (○) and RFL cells (●), respectively.

MuLV was followed by time-lapse cinematography. RFL cells ( $3 \times 10^5$  cells) were plated in a culture flask (TD 40) and infected with Moloney-MuLV. After adsorption for 1 h at 37°C, 5 ml of medium was added to the culture flask. The cells were observed under a phase-contrast microscope and photographed every minute. At 43 h after infection, two neighboring cells fused. Thereafter, the fused cells and other neighboring cells fused sequentially at intervals of approximately 4 h. Similar results were obtained in several separate experiments.

Replication of infectious virus within RFL cells was tested by the XC test. Culture fluid of RFL cells infected with Moloney-MuLV were checked every day for the presence of infectious virions. Only a few plaques were formed by the culture fluid harvested 6 days after infection. Electron microscopically, a small number of C-type viruses were detectable on syncytium in

RFL cultures. However, it is not clear from the present study whether the cell fusion of RFL cells requires a productive MuLV infection or only an abortive infection. Syncytium formation in RFL cultures induced by MuLV may not require synthesis of infectious MuLV, though RFL cells produce a small number of progeny virus. The size of a good many syncytium foci formed in RFL cultures is very small as compared to those in XC assay, suggesting a low sensitivity of RFL cells to productive infection by MuLV.

From the study described above, it appears that RFL cells form syncytia as a cytopathic effect after infection with MuLV. The syncytium foci could be counted as SFU under a microscope, and the number of them was proportional to the concentrations of infected virions. The assay with RFL cells is a sensitive method for direct infectivity assay. It is of interest to determine whether other mammalian C-type RNA tumor viruses (5, 7, 8, 11) induce fusion of RFL cells.

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