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Adaptation of Plaque Assay Methods to the in Vitro Quantitation of the Radiation Leukemia Virus

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A modification of the XC cell procedure for murine leukemia virus assay which yields quantitative data over a wide range of virus concentrations is described. By using serial passage of infected cell cultures and reversal of the plating sequence in the XC procedure, titers of radiation leukemia virus (RadLV) were obtained which were about 10-fold higher than those found by using the conventional assay. By using the modified procedure, it was observed that, even at high multiplicities of infection, less than 10% of the cells function as infective centers, although the proportion increases with serial passage. It was also observed that exposure of infected cells to UV light, which is commonly used to make plaques more visible in the conventional XC cell test, inhibits plaque formation in the RadLV system. Substitution of X irradiation for UV exposure improved plaque visibility without loss of sensitivity.

Radiation leukemia virus (RadLV) is consistently extractable from thymic lymphomas induced in strain C57BL mice by appropriate doses of whole-body X irradiation and may be serially propagated in vivo and extracted from the thymic lymphomas which it induces after inoculation into neonatal or infant C57BL/Ka mice (6, 7). Unlike several of the other murine leukemia viruses, this virus has been notably difficult to propagate or assay in vitro.

About 3 years ago it became possible to assay RadLV by virtue of its helper function for the murine sarcoma virus, and focus assay techniques were successfully developed (2, 3). However, in practice the focus assay proved complex and difficult to reproduce and was therefore not satisfactory for routine use. Recently the simpler and more reproducible plaque assay, based on syncytium formation by rat XC cells after contact with cells infected with murine leukemia viruses, was introduced by Rowe et al. (9). Although we tried the XC cell assay in its originally reported form for the titration of RadLV, the titers observed with this technique proved to be relatively low, and the method was quite insensitive compared with the focus assay or with in vivo bioassay for the same preparations of virus. Accordingly, we have investigated and report herewith certain modifications of the XC cell assay which permit the determination of RadLV titers with greater sensitivity and reproducibility.

MATERIALS AND METHODS

Tissue culture. All of the cell lines and mouse embryo fibroblast (MEF) cells were grown in complete medium (CM) composed of Eagle minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and polymyxin (50 units/ml).

XC cells were kindly provided by A. J. Hackett of the Naval Biomedical Research Laboratory, Oakland, Calif. A continuous line of C57BL/Ka mouse embryo fibroblasts, designated BL-5, and the corresponding RadLV-infected subline, designated BL-5 (RadLV), were established in this laboratory and have been described elsewhere (8). BL-3T3 cells were established recently from C57BL/Ka-MEF cells based on the procedure developed by Todaro and Green (11). Secondary cultures of C57BL/Ka-MEF cells were prepared as described previously (2).

Virus. Wild-type preparations of RadLV were obtained from cell-free extracts of RadLV-induced lymphoma tissue by a previously described procedure (7). Supernatant fluids of 3-day-old BL-5 (RadLV) cell cultures were used as the in vitro source of RadLV.

XC cell assay. Indicator cells (MEF, BL-5, or BL-3T3) were plated in plastic petri dishes (50 mm) at a concentration of 2×10^5 cells per dish. After 24 h the culture fluid was removed, and the dishes were

treated with 25 μg of DEAE-dextran per ml for 1 h at 37 C. Then the DEAE-dextran was removed, and the culture was infected with 0.2 ml of a virus-containing preparation. Serial dilutions of the virus were made in ice-cold CM with 4 μg of Polybrene per ml (Abbott Laboratories) added. After adsorption for 2 h at 37 C, unattached virus was removed, the dishes were rinsed with MEM, and 5 ml of CM was added. Four days after infection, the dishes were irradiated with X rays or UV light, and 10^6 XC cells were added. Medium was replaced every 2 days. Plaque counts were determined on the fourth day after UV irradiation or the sixth day after X irradiation under low-power microscope magnification.

Reverse XC cell test. In a modification of the XC cell assay which reverses the usual plating sequence, the virus-infected cells, at appropriate intervals after infection, were plated over a layer of XC cells. The infected cultures were trypsinized 4 days after virus infection, and samples of 10^2 to 10^5 cells were plated onto dishes which had been seeded with 10^5 XC cells 24 h earlier. Medium was changed on the third day, and plaques were counted on the fourth day. By continued passage of infected cells through a series of subcultures, the reverse XC cell procedure was readily adapted to the end-point dilution assay of low concentrations of input virus.

RESULTS

Plaque formation by RadLV in the XC cell assay; effect of irradiation. In general, as reported by Rowe et al. (9), plaques were not readily seen without UV irradiation of the cultures. This was especially true when the XC cell overlay was performed on the sixth or seventh day after infection of the cultures with RadLV. However, if the overlay with XC cells was carried out on the third or fourth day after infection, when the BL-5 or MEF cells were still in early stationary growth phase, plaques were relatively clear even without irradiation.

Exposure of infected tissues to UV irradiation made the plaques much easier to see but sharply decreased the number of plaques as a function of UV dose (Fig. 1). There appeared to be two components to the slope of the curve for UV inactivation of plaque formation. Plaque number at a UV dose of 1,600 ergs/mm² was only 10% that of the unirradiated control level. It was also observed that the UV-irradiated cells detached from the dishes within 24 h after irradiation.

Attention therefore turned to a study of the effect of X irradiation on the cultures. It was found that a dose of 10 krads yielded plaques which were more clearly delineated than those in the unirradiated control dishes, although not as clearly as the plaques seen after UV irradiation. However, in striking contrast to the effect of UV, X irradiation did not reduce the number

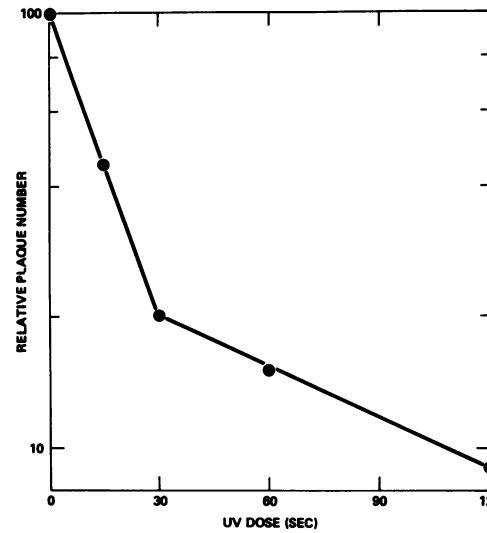

FIG. 1. *Dose-response curve for UV inactivation of plaque formation by RadLV-infected cells in the XC cell assay. Mouse embryo fibroblast cells were infected with in vivo-derived RadLV. Four days after infection, the dishes were rinsed with saline and exposed to UV irradiation with a germicidal lamp at a dose rate of 14 ergs per mm² per s. In this and all subsequent experiments, three plates per point were used. The average number of plaques per plate in the unirradiated samples was normalized to 100 to calculate relative plaque numbers in the UV-irradiated samples.*

of plaques, and most of the cells in the X-irradiated dishes remained attached for at least 6 days after irradiation. Accordingly, X irradiation at a dose level of 10 krads, with inspection of the dishes at 6 days, became the routine procedure for conventional XC cell assays of RadLV. A less satisfactory alternative is to examine plaques at the third or fourth day after XC cell overlay without irradiation.

Plaque formation in the reverse XC cell assay. The infected cells were plated as an overlay on cultures of XC cells, the reverse of the usual sequence. Three to four days later, small, clear plaques composed of multinucleated giant cell syncytia were observed. There was a one-to-one correlation between the number of infected cells and the number of plaques formed (Fig. 2), indicating that 100% of BL-5 (RadLV) cells were infected with RadLV and served as infective centers. Plaque number was not affected by the addition of 10^5 uninfected BL-5 cells to the system at the time of overlay, suggesting that secondary plaque formation is not a significant problem. X irradiation of the BL-5 (RadLV) cells with a dose of 2 krads just before seeding on the XC cell layer did not affect their plaque-

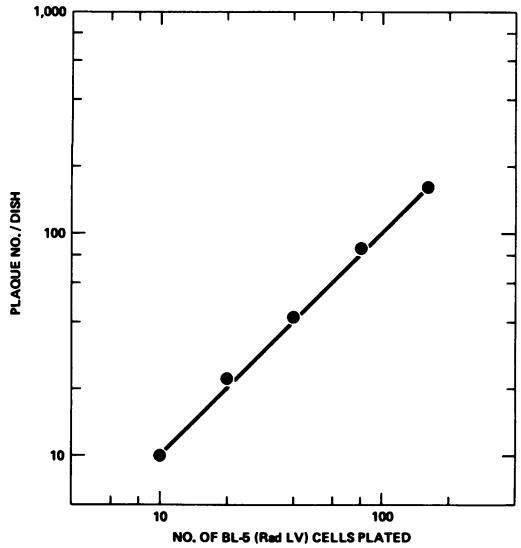

FIG. 2. *Correlation between numbers of infected cells plated and plaque number. Various numbers of BL-5 (RadLV) cells were inoculated as overlays on dishes previously seeded with XC cells. Plaques were counted after 4 days, as described in the text.*

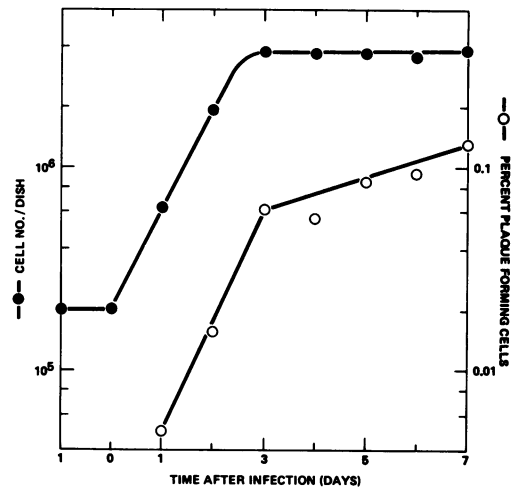

FIG. 3. *Kinetics of cell growth versus plaque-forming cell development after virus infection. BL-5 cells were infected with in vivo-derived RadLV. Each day thereafter, the cells were trypsinized and inoculated as an overlay on dishes previously seeded with XC cells. Symbols: ●, cell-growth curve; ○, percentage of plaque-forming cells.*

forming ability. Accordingly, irradiation is not used in the reverse XC cell assay.

Development of plaque-forming cells after virus infection. The rate of increase in the number of plaque-forming cells in BL-5 cell cultures after infection with in vivo-derived RadLV, as detected by the reverse XC cell assay, is presented in Fig. 3. The percentage of plaque-forming cells per dish increased rapidly while the cells were in the logarithmic phase of growth, and less rapidly thereafter. The response of MEF cells was similar to that of BL-5 cells. At the time of each subculture, the percentage of plaque-forming cells was determined and was found to increase at each passage (Fig. 4). The same data are plotted as a function of serial dilution through each passage in Fig. 5. In the first passage, the percentage of plaque-forming cells was a linear function of virus dilution, but the slope of the curve became progressively flatter through the third and fourth passages when more than 10% of the cells in all dishes had become infected. In three successive experiments, it was observed that dishes which lacked any plaque-forming cells at the end of the second passage remained negative through the third and later passages. Accordingly, a practical end point in the reverse XC cell assay appeared to be the end of the second passage.

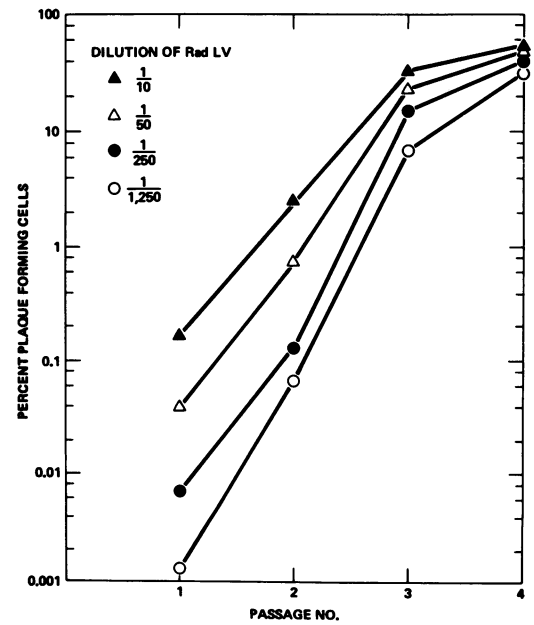

FIG. 4. *Rate of increase of plaque-forming cells with serial cell passage in the reverse XC cell assay. BL-5 cells were infected with different dilutions of in vivo-derived RadLV and assayed by the reverse XC cell procedure at each cell passage for the percentage of plaque-forming cells per dish. The results with four different dilutions of initial input virus are shown.*

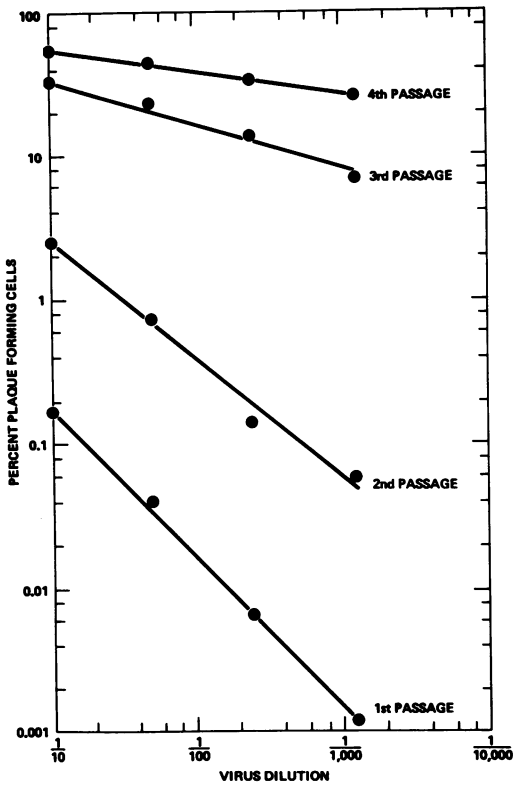

FIG. 5. *Virus titration curves at serial cell passages in the reverse XC cell assay. The percentage of plaque-forming cells at each passage is expressed as a function of dilution of input virus at the first passage. The change in slope with serial passage is evident.*

Comparative sensitivity of the XC cell and the reverse XC cell assays. Typical titration curves for in vivo-derived RadLV on three different cell lines, using the XC cell assay with X irradiation as described above, are seen in Fig. 6. The sensitivity of BL-3T3 cells was consistently about one decade lower than that of either BL-5 or MEF cells.

Virus titers expressed in terms of PFU per 0.2 ml of input virus, as calculated from the titration curves of Fig. 6, are listed in Table 1 as are results of the end-point dilution assay on the same preparation of RadLV, performed at the same time with the reverse XC cell technique. Although the number of dishes used in the reverse XC cell assay was small, it is clear that the end-point dilution titer by the reverse XC cell method was significantly higher than the PFU titer by the conventional XC cell assay on BL-5 and MEF cells, though not on BL-3T3 cells. This same comparison has been repeated several times with BL-5 and MEF cells, and a consistently higher (usually 1 to 2 $\log_{10}$) end-point dilution titer has been observed with the reverse XC cell procedure than with the PFU titer yielded by the XC cell assay. The end-point dilution titer of the in vitro-derived virus from cultures of BL-5 (RadLV) cells was consistently in the range 5×10^7 to 5×10^8 PFU/ml.

DISCUSSION

Plaque formation after the overlay of infected mouse cells directly on cultures of XC cells was previously noted by Johnson et al. (5). Amplification of the sensitivity level for the detection of very small amounts of input virus by serial passage of infected cells has been successfully applied for the detection of murine leukemia virus in the complement fixation assay (4) and the interference assay (10). Combining these two techniques, reversal of plating sequence and serial passage of infected cell cultures, yielded an end-point dilution plaque assay.

The reverse XC cell plaque assay has the advantage of providing quantitative data over a very wide range of virus concentrations. Whereas the standard XC cell method cannot be used to study cells infected with high concentrations of murine leukemia virus, because under these conditions the plaques become confluent, the reverse plaque assay procedure permits small samples of the heavily infected

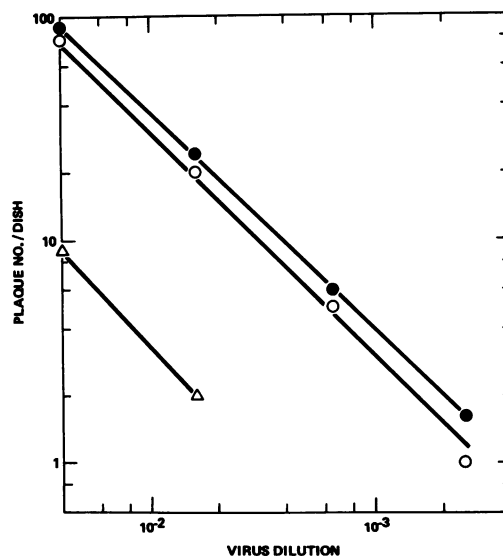

FIG. 6. *Titration curve with the modified XC cell assay on three different indicator cell lines. Four days after infection, the dishes were exposed to 10 krads of X irradiation and inoculated with an overlay of 10^6 XC cells. Plaques were counted on the sixth day thereafter. Symbols: ●, BL-5 cells; ○, BL-MEF cells; △, BL-3T3 cells.*

cell population to be plated as an overlay. Thus, this procedure yields readily countable numbers of plaques from which the percentage of plaque-forming cells in the original population may be calculated. It therefore becomes possible to detect the existence of a saturation plateau at high multiplicities of infection, at which 100% of the cells should be infected. By use of this test, we have indeed observed a saturation plateau for RadLV on BL-5 and MEF cells; to our surprise, it occurred when only about 10% of the exposed cell population had become plaque-forming units, no matter how high the virus input (Fig. 7). This observation is being studied in greater detail and will be described more fully in a later publication.

Conversely, the end-point dilution procedure using the reverse XC cell technique provided a more sensitive method for the detection of very small amounts of RadLV. However, relatively large statistical errors are inevitable with the end-point dilution titration, and thus a more quantitative and reliable assay is required for routine use. The XC cell assay, as modified by the use of X-ray rather than UV irradiation, appears to serve this purpose for RadLV and may also prove useful for other murine leukemia viruses.

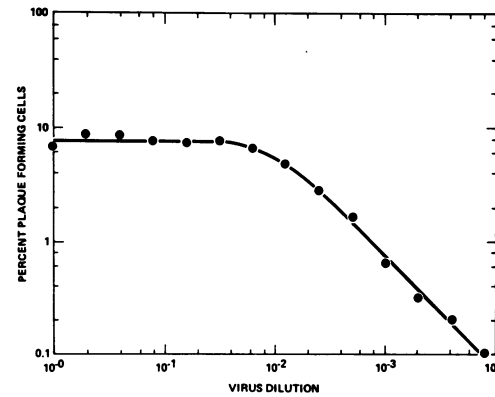

FIG. 7. *Plaque-forming cells at high multiplicities of infection with RadLV. BL-5 cells were infected with serial twofold dilutions of in vitro-derived RadLV. The number of plaque-forming cells was determined by the reverse XC cell assay. Note the saturation plateau at about 8% of the total cell population.*

TABLE 1. *Comparative sensitivity of the XC cell and reverse XC cell procedures for the assay of radiation leukemia virus*

XC cell test		Reverse XC cell test[a]	
Cell line	Virus titer: 0.2 ml	Virus dilution	Positive dishes: total dishes
BL-3T3	360 PFU	1:40	3:3
		1:160	3:3
		1:640	1:3
		1:2,560	0:3
		Control	0:3
BL-MEF	3,160 PFU	1:40	3:3
		1:160	3:3
		1:640	3:3
		1:2,560	3:3
		1:10,240	2:3
		1:40,960	2:3
		1:163,840	0:3
		Control	0:3
BL-5	3,400 PFU	1:40	3:3
		1:160	3:3
		1:640	3:3
		1:2,560	3:3
		1:10,240	2:3
		1:40,960	2:3
		1:163,840	0:3
		Control	0:3

[a] Two serial passages of infected cells were made at 4-day intervals before overlay on XC cells for detection of plaque-forming cells.

The fact that the end-point dilution titer observed by the reverse XC cell technique was consistently higher than the PFU titer obtained with the XC cell assay suggests that the reduction of plaque number after UV irradiation in the XC cell test was not due to prevention of satellite plaque formation but rather to interference by UV with the process of plaque formation. The D_0 value of the UV-sensitive component of the dose response curve (Fig. 1) is 616 ergs/mm^2, a value which is compatible with the D_0 for UV inactivation of virus production by murine sarcoma-leukemia virus complex carrier cells, as reported by Yoshikura (12). The ultraviolet dose commonly used for the XC cell assay in other laboratories reportedly varies from 1,400 ergs/mm^2 to 1,800 ergs/mm^2 (1, 9). This UV dose level was reported to have no effect on plaque number in the systems tested. However, in the case of RadLV tested on BL-5 and MEF cells, this dose reduced the plaque number to 10% of the unirradiated control level. It is not yet known whether this striking sensitivity to UV is a property of the virus or of the cell lines used in these studies.

ACKNOWLEDGMENT

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