MIXED CULTURE CYTOPATHOGENICITY: A NEW TEST FOR GROWTH OF MURINE LEUKEMIA VIRUSES IN TISSUE CULTURE

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Abstract.—Tissue culture cells of a Rous virus-induced rat tumor undergo syncytium formation when placed in contact with mouse embryo cell cultures infected with murine leukemia viruses. This phenomenon can be used as a cytopathic end point for isolating and titrating these viruses in tissue culture. The principle should be applicable to detection of leukemia viruses of other species.

Sensitive tissue culture techniques have been developed for the isolation and propagation of murine leukemia viruses.[1, 2] However, the growth of these viruses in cell culture is not accompanied by any observable change in the appearance of the cells; consequently, demonstration of their presence in a culture requires use of serological procedures—either complement fixation (the COMUL test)[1, 2] or fluorescent antibody.[3]

This report describes a unique endpoint system for detecting the growth of murine leukemia viruses in cell cultures. It was observed that a cell line of a rat tumor induced by an avian sarcoma virus, when added to mouse embryo cell cultures infected with murine leukemia viruses, developed rapid and striking cytopathic effects. This phenomenon, referred to here as mixed-culture cytopathogenicity, has been utilized for the development of an assay system.

Materials and Methods.—Viruses: Tissue culture passaged lines[1, 2] of various laboratory and recently isolated field strains of murine leukemia virus were used. As a representative naturally occurring virus, pooled serum of 6-month-old AKR/N mice was also studied.

Tissue cultures: Cell cultures of 14- to 17-day NIH Swiss and BALB/cN mouse embryos were prepared and maintained as previously described.[1, 2]

The indicator cells were the XC line of Svoboda *et al.*[4, 5] This cell line was established from a Wistar rat tumor induced with the Prague strain of Rous sarcoma virus, and has been carried *in vitro* for a large number of passages. The cells contain the Rous virus genome, as determined by rescue procedures, but do not appear to release infectious virus. Tests of the line for mycoplasma and murine viruses were negative.

All cultures were grown and maintained in medium consisting of Eagle's minimal essential medium with 10% unheated fetal calf serum, 2 mM glutamine, 250 U/ml penicillin, and 250 mcg/ml streptomycin.

Procedure for virus detection: Test tubes and 60-mm Falcon plastic Petri dish cultures were seeded with 25,000 and 350,000 mouse embryo cells, respectively, and were inoculated with virus on the following day. The cultures were held at 36°C, and fluids were changed twice weekly. At various times thereafter, usually 14 or 21 days, longitudinal strips were scraped out of the cell sheet with the tip of a capillary pipet, and freshly trypsinized XC cells were added to the culture fluid; tubes were overlaid with 4×10^5, and Petri dishes with 10^6 XC cells. The cultures were then held at 36°C for 1 day and then usually placed at 32°C. They were observed daily and discarded on the 7th day after adding XC cells.

Results.—Characteristics of the mixed-culture cytopathogenicity: The XC cell is a rapidly growing cell line of relatively small cells which appear triangular, broad, fusiform, or round. They attach well to glass and may develop areas of piled-up round cells. On occasion, a few large round cells containing one or two large vacuoles can be seen; syncytial areas have never been seen in control cultures.

When seeded onto uninfected mouse embryo cell cultures, the XC cells settle into the cell sheet and the scratched channels. They multiply rapidly, becoming confluent in the channels and invading and replacing the cells in the sheet. Within five to seven days, most of the mouse cell sheet has been destroyed and replaced by the rat tumor cells.

When overlaid on mouse embryo cells infected with mouse leukemia viruses, the XC cells show three types of alteration. The earliest response is formation of syncytial areas containing from four to several dozen nuclei; syncytium formation can be detected within 3 to 6 hours after addition of XC cells and is generally extensive by 24 hours (Fig. 1). The syncytia are most readily seen in the channels, where they usually appear adjacent to the margins of the mouse cell sheet. Small syncytia also form in the cells lying on or in the cell sheet, but these are difficult to visualize in unstained preparations.

The syncytial stage is followed by formation of large numbers of bizarre, giant round cells with one or two large vacuoles (Fig. 2); they probably represent syncytia which have undergone rounding. These develop at two to three days and are most prominent in cultures held at 32°.

The third effect is most readily noticeable at about one week and consists of a dramatic preservation of the integrity of much of the mouse cell sheet, which has been replaced by the XC cells in control cultures.

Identical changes were observed when XC cells were overlaid onto rat embryo cell cultures infected with murine leukemia viruses.

Sensitivity to various strains of murine leukemia virus: To evaluate whether the XC cells would respond to a wide range of virus strains, a battery of murine leukemia viruses of various host range patterns were inoculated into tube cultures of NIH and BALB/c mouse embryo. The inocula were diluted to contain about $10^{4.5}$ $TCID_{50}$ per culture as measured by the COMUL test. Several other murine viruses were also included as controls. The mouse embryo tubes were scratched and seeded with XC cells at nine days. The results are shown in Table 1.

In all instances, the mouse embryo cultures supporting the growth of the leukemia virus induced cytopathic effects in the XC cells; where the mouse embryo culture was resistant to the leukemia virus strain, no such effects were induced. Also, the other murine viruses tested gave negative results.

To test for the sensitivity of XC cells to direct infection by leukemia viruses, the agents listed in Table 1 were inoculated onto coverslip cultures of XC cells. Little or no syncytium formation was seen. The cover slips were fixed with acetone on the sixth day and tested by the indirect fluorescent antibody test, with group-reactive rat antiserum[3] against Moloney murine sarcoma virus. A small number (<1%) of cells showed specific fluorescence in the cultures inoculated with the Moloney, Gross Passage A, and Kirsten viruses, while the others were negative. It should be noted that XC cells inoculated with $10^{4.5}$ $TCID_{50}$ of

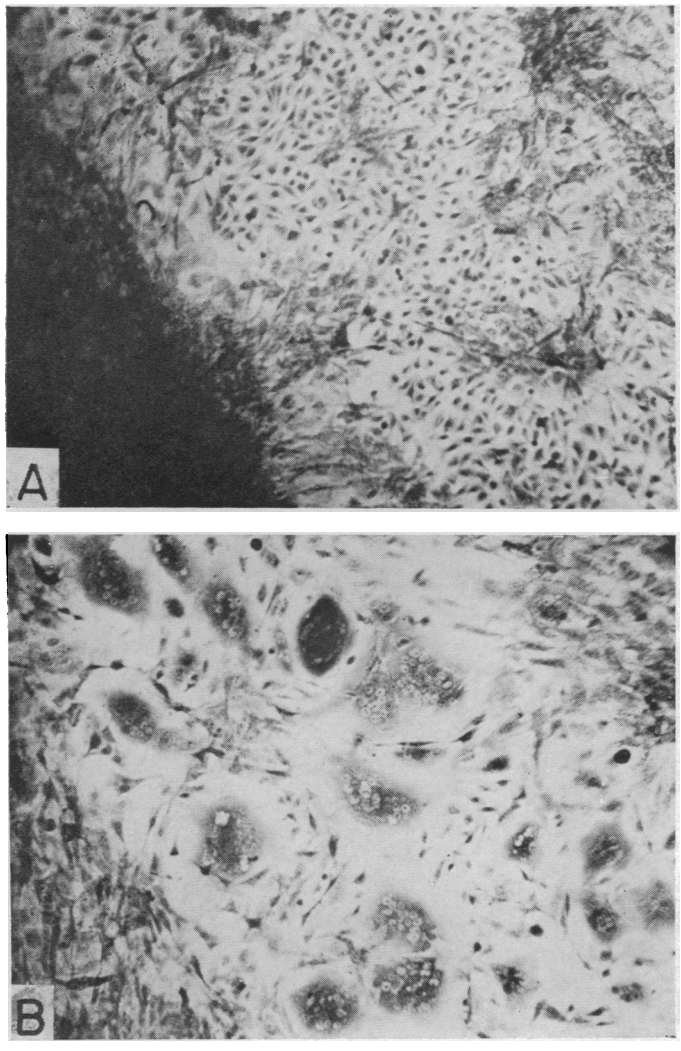

FIG. 1.—NIH-ME 11 days after planting and 1 day after addition of XC cells, Giemsa stain, X92. The fields show XC cells in a scratched area with the NIH-ME cell sheet at two corners. (*A*) Uninoculated control culture; (*B*) culture inoculated with Moloney leukemia virus on day 1.

Gross Passage A virus developed, after one to two weeks, a moderate degree of syncytial and giant cell cytopathogenicity, along with complement-fixing antigen. It thus appears that the XC cell has a relatively low susceptibility to infection and syncytium formation by various leukemia viruses, which can be enhanced by placing them in contact with virus-producing cells, analogous to the Visna virus system described by Harter and Choppin.[7]

Comparative sensitivity of the mixed-culture test and other tests for presence of virus: The sensitivity of virus titrations in NIH-ME cells with the XC cell cytopathogenicity as end point was compared with that of titrations in which the induction

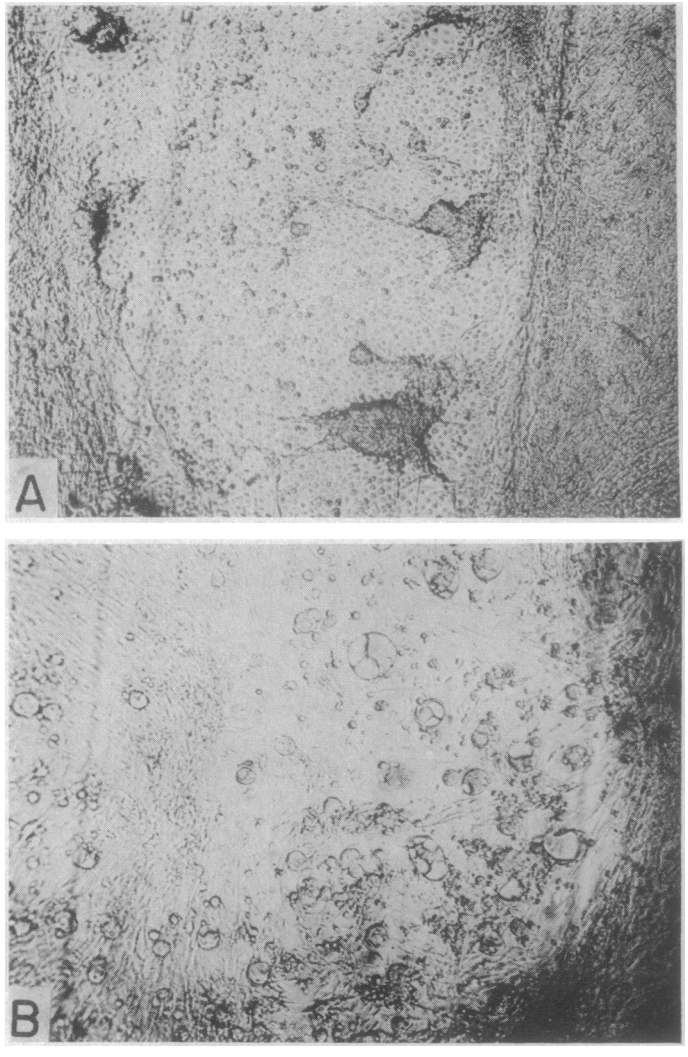

FIG. 2.—NIH-ME 20 days after planting and 4 days after addition of XC cells, unstained, X52. The NIH-ME cell sheet is seen along the sides of the field. (*A*) Uninoculated control culture; (*B*) culture inoculated with Moloney leukemia virus on day 1.

of complement-fixing antigen was the end point (COMUL test). Replicate samples of three virus preparations were titrated in the mixed-culture test (4 tubes per dilution) and in the COMUL test (2 plates per dilution). XC cells were added to two of the tubes on the 14th day after virus inoculation, and to the others on the 21st day; the plates were harvested individually for complement-fixation testing on the 21st day. The results are shown in Table 2. The titers of the Moloney virus preparation were comparable in the two tests, although the Gross Passage A virus gave a somewhat lower titer in the tests for mixed-culture cytopathogenicity. On the other hand, the field specimen of AKR virus gave a significantly higher titer in the mixed-culture test. This difference was doubtless due to the

TABLE 1. *Sensitivity of mixed-culture cytopathogenicity test for detection of various viruses of the murine leukemia/sarcoma complex.*

Virus Group	Strain	Host range for NIH(N) and BALB/c (B) mouse embryo (COMUL test)	Mixed culture Cytopathogenicity Result* NIH-ME	BALB/c-ME
Murine leukemia/sarcoma complex	Moloney	N, B†	3+	4+
	Friend	N, (B)	3+	2+
	Rauscher	N, B	2+	2+
	Gross Passage A	N, (B)	3+	3+
	AKR-L1	N, (B)	3+	2+
	Pope WM1-B	N, B	3+	2+
	Kirsten MEV	N, (B)	2+	±
	C57BL-MCT1	B	—	2+
	BALB/c-D1	B	—	2+
	BALB/c-S1	N, (B)	2+	± (focal)
	MSV(O) (Ting)[6]	—	—	—
LCM			—	—
SV5			—	—
MHV-S			—	—
Sendai			NT	—
NIH-ME control fluid			—	—

NT: no test.
* Graded on — to 4+ scale based on proportion of XC cells showing cytopathic effects.
† N and B mean that the virus grows well in NIH-ME and BALB/c mouse embryo, respectively. (B) means limited replication in BALB/c-ME.

TABLE 2. *Comparison of mixed-culture cytopathogenicity and* COMUL *tests for titration of murine leukemia viruses.*

Virus	Titer ($TCID_{50}$/0.1 ml) as determined by: Mixed-Culture Cytopathogenicity XC cells added on 14th day	XC cells added on 21st day	COMUL test (21st day)
Moloney leukemia virus	$10^{5.5}$	$10^{4.5}$	$10^{4.8}$
Gross Passage A virus	$10^{3.5}$	$10^{3.5}$	$10^{4.5}$
Serum of AKR mice	$10^{1.0}$*	$10^{1.5}$*	Neg†
Control NIH-ME TC fluid	Neg	Neg	

* Focal changes in all tubes.
† Titer of $10^{0.5}$ as determined by blind passage.

failure of this virus to disseminate through the culture; the mixed-culture test could detect the focal changes, while blind passage was required for the COMUL test to become positive.

NIH-ME cultures inoculated with Moloney leukemia virus that had been exposed to a dilution of rat antiserum containing eight units of neutralizing antibody did not give a positive response with XC cells.

Discussion.—The cytopathogenicity system described here should provide a useful technique for detecting murine leukemia viruses. Since it does not require serological reagents, which are difficult to produce and possibly variable in the spectrum of antibodies contained,[1, 2] the test may be more broadly useful than the COMUL test. Also, it should prove more sensitive than the COMUL test in being able to detect small focal areas of infection and should be adaptable for use as a quantal assay system.

In preliminary studies, the mixed-culture cytopathogenicity system has proved to be sensitive for detecting murine leukemia viruses in several chronically infected mouse and rat cell lines, as well as in the acute infections described here.

The mechanism of syncytium induction in the XC cell is not known. From the rapidity of their formation it can be inferred that the syncytia arise by cell fusion rather than endomitosis; also, mitotic figures have not been seen in the syncytia. Proximity to infected cells seems to be a major factor; virus alone may induce a few syncytia and giant cells, but they form after several days rather than within hours. Also, preliminary studies with fluorescent antibody techniques indicate that syncytium formation occurs immediately adjacent to foci of infected mouse embryo cells. Whether the release of virus particles, the presence of viral antigen in the surfaces of mouse embryo cells, or some other cellular alteration is the stimulus for syncytium induction is not known. Also, it is not known whether the mouse cells form part of the syncytial masses.

The protection of the infected mouse embryo cell sheet against invasion and destruction by the XC cells is often dramatic. This may result from the damage to the XC cells which settle onto the infected cells, or there may be a form of contact inhibition not induced by mouse cells with normal plasma membranes.

The XC cell seems to be unique in its responsiveness; another Prague strain Rous rat tumor cell line, a variety of murine leukemia infected or transformed rat and hamster cells, and normal rat cells have shown no alterations when added to murine leukemia infected NIH-ME. Also, the XC cell showed no morphologic alteration when plated on chick cells infected with an avian leucosis virus.

The mixed-culture cytopathogenicity technique represents the first end point for bioassay of a nonfocus-forming leukemia virus which does not require *in vivo* studies, serologic reagents, or electron microscopy. Thus, the principle may be useful in searches for leukemia viruses of other species.

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