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Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma

(AKR mice/*Akv-2* congenic/nonproducer cell line/pseudotype)

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ABSTRACT **Murine leukemia viruses capable of malignant transformation of mink tissue culture cells have been isolated from an AKR thymoma cell line and from a spontaneous reticulum cell sarcoma in an NIH Swiss mouse partially congenic for the AKR ecotropic virus-inducing locus *Akv-2*. In contrast to the recently described mink cell focus-inducing strains of murine leukemia virus, at least one of the two transforming strains is replication defective. Nonproducer mink cells carrying the genome of the transforming virus of AKR origin have been isolated, and pseudotype transforming viruses generated.**

We have recently reported the detection of a unique category of murine leukemia virus (MuLV) in the thymuses of preleukemic and leukemic AKR and other high ecotropic MuLV strains of mice (1). These viruses are recognized by their ability to produce cytopathic foci in a tissue culture line of mink lung cells, and are presently referred to as MCF (mink cell focus-inducing) strains. Their biological features suggest that they represent genetic recombinants between ecotropic and xenotropic MuLVs.

In addition to these strains, whose effects are in large part cytolytic, we have encountered two strains of mink-infectious virus that produce malignant-type transformation of the mink cells. These viruses were isolated from high-MuLV inbred mice with spontaneous hematopoietic neoplasms. One of the strains, from an AKR lymphoma, is replication defective, allowing the selection of a transformed nonproducer clonal cell line from which transforming pseudotypes can be obtained. These transforming viruses may play a role in the development of spontaneous virus-induced murine tumors.

MATERIALS AND METHODS

Cells and Virus Assays. Lymphocyte lines were maintained as suspension cultures in EHAA medium (2) supplemented with 10% heat-inactivated fetal calf serum, penicillin/streptomycin/glutamine, and 5×10^{-5} M mercaptoethanol. Established cultures were split 1 to 2 every 2–3 days.

The mink lung cell line (ATCC no. CCL-64) (3) was used in assays for transformation and fluorescent antibody (FA) focus induction. The cells were maintained on 10% heated fetal calf serum and penicillin/streptomycin/glutamine in Dulbecco's modified basal medium. Inoculations were as described (4), except that polybrene (Abbott; 16 μg/ml for the first day of infection) rather than DEAE-dextran was used for transformation assays with cell-free virus. Infectious center assays with mitomycin C-treated cells, and FA focus induction tests were done as described (4).

Agar Assay. Assay for growth of cells in semisolid medium was based on the method of Macpherson (5). Cells were trypsinized and suspended at the desired concentration (usually 1 to 2×10^6 cells per ml) in 1 ml of tissue culture medium. Two milliliters of agar medium containing 10% heated fetal calf serum, penicillin/streptomycin/glutamine, and 0.5% agar or agarose in Eagle's minimal essential medium was mixed with the cells and layered over 7 ml of hardened 0.5% agar medium in a 60-mm petri dish. The cells were fed with 3 ml of 0.3% agar medium at 5–7 days.

Transformed clones were established by picking colonies from agar with a pasteur pipette under microscopic visualization. The colony-containing agar plug was placed in a 35-mm petri dish containing 1 ml of tissue culture medium. The colony was teased free from the agar with sterile needles, and a further 1 ml of medium was added. Visible clusters of adherent cells appeared in 5–7 days. The clonal lines were carried in Eagle's minimal essential medium with 10% heated fetal calf serum and penicillin/streptomycin/glutamine.

Abbreviations: MCF, mink cell focus-inducing; MuLV, murine leukemia virus; FA, fluorescent antibody.

RESULTS

Isolation of transforming viruses

T-8 Strain. The initial isolate was from an *in vitro* thymoma cell line, AKT-8, from a spontaneously lymphomatous AKR/J mouse. The massively enlarged thymus was minced and a portion suspended in EHAA medium. Large tissue fragments were allowed to settle for 10 min, and the supernatant was transferred to a 75-cm^2 tissue culture flask. After the lymphocytes were well established in culture, the cells in the culture supernatant were transferred at intervals to fresh flasks to eliminate the adherent cell population. These cells have been maintained as a suspension culture for 12 months.

Virus characterization studies of the thymoma cells were carried out before and after culture. The tumor cells, plated as mitomycin C-treated infectious centers, gave 10^4 XC plaques per 10^6 input cells on SC-1 cells, $10^{1.9}$ foci per 10^6 cells on mink S^+L^- cells (6), and $\geq 10^3$ FA focus-inducing units per 10^6 input cells on mink lung cells. On passage, the infected mink cell culture showed typical MCF virus cytopathic changes, and an MCF virus was subsequently established in cell-free passage. The thymoma culture line has continued to produce significant amounts of viruses infectious for SC-1 and mink cells (though not registering efficiently as XC plaques in the SC-1 cells) and smaller amounts of virus registering as mink S^+L^- foci; on one occasion, MCF-type virus was again isolated.

Surprisingly, mink cell cultures inoculated with mitomycin C-treated AKT-8 tissue culture cells or filtered culture fluid developed many areas of morphologically altered cells resembling cell transformation. These foci generally appeared 8–10 days after inoculation, and were thickened, somewhat granular areas whose edges were often sharp, with a "rolled" appearance; some foci contained large ovoid or round cells, but it was generally difficult to distinguish individual cells in the thickened areas. Unlike the previously described MCF foci (1), these foci showed no pycnotic cells, vacuolization, or cell loss (Fig. 1).

The focus-inducing activity was initially detected 3 weeks after establishment of the culture and has since been detectable in the original culture on several occasions, including after 10

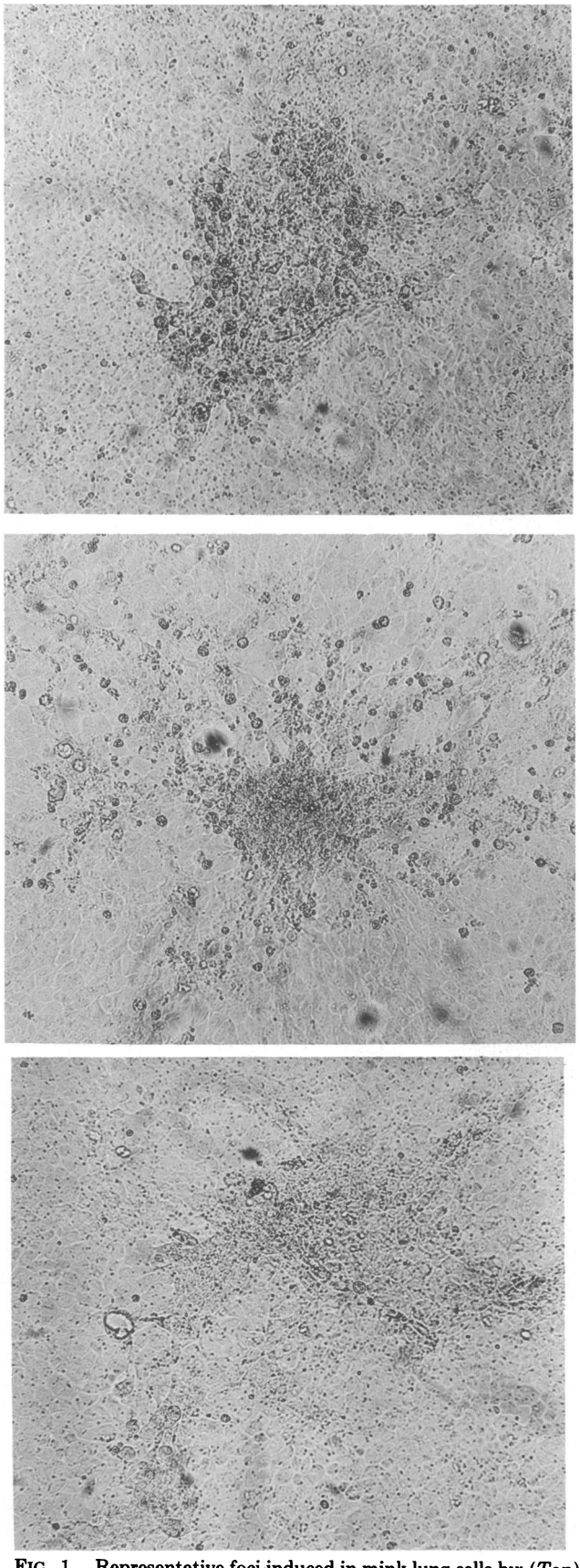

FIG. 1. Representative foci induced in mink lung cells by: (*Top*) T-8 virus, (*Middle*) MCF 247 virus, and (*Bottom*) M-44 virus. (×62.)

months of culture *in vitro*. We have designated the focus-inducing virus from the AKT-8 culture T-8 virus.

Quantitative studies on T-8 virus have been hampered by the lack of a sharp assay system; foci in mink cells are often indistinct and difficult to score, and frequently cell transfer was required for clear transformation to be observed. Undiluted culture fluid from the AKT-8 culture generally produced several hundred foci in the mink lung cells, but dose-response relations are not clear. Titration in mink cells of an amphotropic pseudotype of T-8, the derivation of which is described below, revealed apparent single-hit kinetics. Other cell lines have been inoculated with T-8, including a variety of mouse cell lines and the NRK line of rat kidney cells; although some cultural alterations were occasionally seen, none of the cultures has yet shown conclusive focus induction.

M-44 Strain. A 17-month-old mouse partially congenic for the *Akv-2* ecotropic MuLV-inducing gene of AKR, on an NIH Swiss genetic background, developed a spontaneous neoplasm of the spleen and lymph nodes, as well as a pulmonary adenoma. The hematopoietic neoplasm showed histologic features of reticulum cell sarcoma. Lymph node cells were treated with mitomycin C and plated as infectious centers. On SC-1 cells they produced $10^{3.8}$ XC plaques per 10^6 cells; on mink S^+L^- cells, $10^{1.9}$ foci per 10^6 cells; and on mink lung cells, about $10^{3.1}$ FA foci per 10^6 cells plated. The recipient mink cell cultures were transferred serially; the first transfer cells were uniformly positive for MuLV antigen by FA testing, and the cultures showed suspicious areas of transformation. The second transfer cells showed large numbers of distinct foci resembling those seen with T-8 virus (Fig. 1). On further passage the line has shown confluent transformation. Plating the cells as mitomycin C-treated infectious centers on mink cells induces large numbers of transformed cell foci. Serial passage of cell-free culture fluids through fresh mink cell cultures has also produced transformation, but in low titer ($\leq 10^3$ focus-forming units per ml), while the titer of virus that induces foci in the mink S^+L^- cells is about 100-fold higher.

Cells from the neoplastic lymph nodes carried *in vitro* yielded a mixed culture of fibroblastic and macrophage-like cells. This culture has yielded ecotropic and mink-infectious viruses, including an MCF strain, but transforming virus has not been detected.

Growth of virus-inoculated mink cells in agar

The ability of tissue culture cells to form colonies in semisolid medium has been correlated with their malignant potential *in vivo* (7–9) and has been used as an indicator of transformation by both RNA and DNA viruses. Mink cells infected 11 days previously with a filtrate of AKT-8 cell culture fluid and showing many foci were trypsinized and plated in 0.33% agar. Within 6 days, multiple large colonies had been formed by the virus-infected mink cells; none were produced by the uninoculated control cells. Similarly, mink cell cultures showing foci of transformation after infection with the M-44 virus formed multiple large colonies when suspended in agar. In contrast, cells infected with a prototype MCF strain, MCF 247 (1), did not form agar colonies.

Selection of transformed nonproducer mink cells and pseudotype formation

Three agar colonies from mink cells infected with AKT-8 culture fluid were picked and established as continuous cell lines. Their morphology differs from the parental mink line; the cells are large and triangular, and there are frequent bizarre multinucleated cells. In confluent cultures there is more piling up and disorganization of the cell sheet than in the normal mink lung cultures. All three lines were negative for MuLV antigen expression, as determined by FA tests on acetone-fixed cells, and the culture supernatants failed to induce foci of transformation, or FA foci, in indicator mink cells. One line, no. 139, which has been the prototype line for virus rescue studies, was also tested for reverse transcriptase activity in culture fluids and

for MuLV p30 reactive in radioimmunoassay, with completely negative results. These results suggest that the T-8 virus genome is defective for both replication and viral p30 antigen production.

We have been able to rescue the T-8 genome from the three nonproducer mink cell lines by superinfection with xenotropic [strain BALB IU-1 (10)] and amphotropic [strain 4070-A (10)] MuLVs. Filtered supernatants from the superinfected cultures, harvested 14 days after inoculation, were tested on mink cells. Both the amphotropic and xenotropic harvests from each of the three transformed mink clones produced foci of transformation; the most active amphotropic pseudotype, from line 139, gave a titer of approximately $10^{3.7}$ focus-forming units per ml, and an about 100 times higher titer of amphotropic virus. Amphotropic and xenotropic MuLVs grown in normal mink cells showed no focus-forming activity. Further, mink cells inoculated with the amphotropic pseudotype gave colony formation in agar when the cells were trypsinized and suspended in agar at 3 or 8 days after inoculation.

DISCUSSION

The studies presented here indicate that, in addition to the primarily cytopathic MCF viruses, viruses with cell-transforming capacity can emerge in hematopoietic neoplasms of high ecotropic virus mouse strains. Their relationship to the MCF strains and their appropriate taxonomic designation are far from clear. The transforming isolates resemble the MCF strains in their origin and in their apparent specificity for the mink lung cell line as an indicator cell in which visible alterations are produced. As working definitions, we consider MCF strains to be those that produce changes which include distinct cytopathogenicity, while those that produce only transformation are considered a distinct, presently unnamed, category. The distinction may not be absolute. We have found that MCF strains display considerable heterogeneity in the type of alteration produced in the mink lung cell cultures; different isolates produce foci with sharply different proportions of pycnosis, thickening of the cell sheet, and vacuolization. Indeed, some isolates produce only pycnosis, and one isolate induces only vacuolization. The transforming strains may thus represent one extreme of a spectrum of recombinant viruses produced in high-virus mice. In particular, the thickening of the cell sheet that accompanies the pycnotic changes with many of the MCF strains (including the prototype strain 247) suggests such a relationship.

It is possible that replication defectiveness may set the transforming strains apart; of the several MCF strains studied to date, all appear to be nondefective, while the one transforming strain that we have been able to study in this regard appears to be replication-defective. Obviously, many more strains will have to be characterized before valid inferences can be made.

Characterization of the transforming strains presents a number of difficulties. The most potent sources of virus have been the AKT-8 thymoma cell culture and the mink culture chronically infected by exposure to the mitomycin C-treated M-44 reticulum cell sarcoma cells. These cultures produce complex populations of viruses, which presumably include ecotropic, xenotropic, MCF, and phenotypically mixed particles, as well as the virions with transforming genomes. In addition, infection by the M-44 transforming virus is accomplished much more readily by infectious center plating than with cell-free virus, which further hinders its separation from other components of the mixed population. In the case of T-8 virus, the isolation of the transforming genome in the mink nonproducer cell lines should allow its biological and biochemical characterization as with other replication-defective transforming viruses. In particular, these cell lines should make it possible to determine if the T-8 genome arose by recombination, as appears to be the case with the MCF strains, certain murine sarcoma viruses, and the Friend spleen focus-forming virus (12, 13).

We do not know if similar genomes will be frequently detectable in lymphomas of AKR and other high-ecotropic virus mouse strains, and many technical problems will have to be solved before this can be effectively studied. We have tested three additional lymphomas by the same tissue culture and infection procedures as with the AKT-8 thymoma line, with negative results. However, these lymphocyte cell lines produced little or no mink-infectious virus, as contrasted with the large amount produced by AKT-8; thus, the failure to detect transforming virus could be due to an insufficient amount of appropriate helper virus rather than absence of transforming virus. Perhaps superinfection with amphotropic virus or induction of endogenous virus by 5-iododeoxyuridine would allow detection of a transforming genome. Other technical problems are that an excess of MCF virus might prevent detection of transformation and that transfer of the infected cultures is frequently needed for clearcut foci to develop.

It is an obvious hypothesis that transforming genomes such as described here may play an immediate role in hematopoietic cell transformation *in vivo*, with ecotropic, xenotropic, and MCF viruses being precursors. This will not be easy to prove or disprove. In preliminary studies (W. P. Rowe and J. W. Hartley, unpublished data) the MCF strain 247 has been found to be highly lymphomagenic in the AKR acceleration test (11). Whether transforming viruses emerge in these induced lymphomas and whether the T-8 pseudotypes are leukemogenic are presently under study.

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