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Generation of oncogenic type C viruses: Rapidly leukemogenic viruses derived from C3H mouse cells *in vivo* and *in vitro*

(mouse leukemia/endogenous type C virus/transforming viruses/MCF viruses)

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ABSTRACT A type C virus was isolated from C3H/10T1/2 mouse cells in culture after activation with iododeoxyuridine. This virus was poorly infectious for mouse cells and did not cause tumors upon inoculation into newborn NIH Swiss mice. Variants with increased infectivity for mouse cells were then derived both *in vivo* and *in vitro* by selecting for variants able to grow to high titers. The highly infectious variants were found to induce mouse fibroblasts to grow in soft agar. When the viruses were inoculated into newborn NIH Swiss mice, 100% of the animals died of leukemia within 4 months. Solid tumors developed at the injection site. Both mouse-tropic and dual-tropic viruses were isolated from the leukemic mice and plaque purified. The first group of viruses produced large syncytial plaques on rat XC cells and did not grow in mink cells. The viruses of the other group replicated well in both mouse and mink cells, producing morphologic changes similar to transformation but not XC syncytia; they, therefore, are members of the newly described MCF class of mouse type C viruses. Isolates from either group were highly leukemogenic on retesting, the mean latent period being 67 days for a mouse-tropic virus and 105 days for one of the dual-tropic viruses. The results led to the conclusion that the better a mouse type C virus grows in cell culture the more effective it is as a leukemogen. Further, it is possible to start with a weakly infectious, nonleukemogenic virus and to convert it to a rapidly replicating, highly leukemogenic virus by passage either in cell culture or in the animal. The availability of a defined series of viruses from a low-leukemia mouse strain that differ greatly in their biologic properties should facilitate studies of the molecular basis for the acquisition of type C virus oncogenicity.

Highly oncogenic type C viruses that produce leukemias or sarcomas in infected animals generally have had a long passage history and, in some cases, their origins are difficult to trace. The development of oncogenic viruses from low or nononcogenic viruses through a defined passage history should facilitate an understanding of the mechanisms by which transforming type C viruses are generated and allow the identification of virus expression associated with induction of neoplasia.

In the present studies, a poorly infectious, XC test negative, N-tropic nontransforming virus isolated from cells of a low-leukemic mouse strain was used to derive rapidly replicating transforming variants under defined conditions, both *in vivo* and *in vitro*. The replication of the parent endogenous mouse-tropic virus, isolated from the C3H/10T1/2 cell line, is blocked at a level prior to the assembly of complete infectious virus. The new viruses replicate efficiently in cell culture and induce lymphocytic leukemia at high incidence and with a short latency period following inoculation of newborn NIH Swiss mice. Certain clones of these new viruses also transform mouse fibroblasts, NIH/3T3 and C3H/10T1/2 (1), and mink epithelial cells in culture.

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MATERIALS AND METHODS

Animals. NIH Swiss mice were obtained from the National Institutes of Health colony. Newborn mice were injected intraperitoneally with freshly cloned virus grown in C3H/10T1/2 CL8 (1) or SC-1 (2) cells; 2000–5000 infectious units (see below) were used per mouse. The animals were weaned at 4 weeks and portions of the tails were removed at monthly intervals for determination of the virus status of the injected animals.

Cells. All cell lines were maintained in Eagle's minimum essential medium (MEM) with 10% heat-inactivated fetal calf serum. The origin and characteristics of the cell lines used for virus growth and virus assays have been described (3, 4). The C3H/10T1/2 CL8 line was developed from a C3H/Heston mouse embryo (1). This strain has a low "spontaneous" leukemia incidence. The 10T1/2 line has been widely used for chemical carcinogenesis studies and has been shown to have endogenous type C viruses that can be activated (3). Ecotropic virus was assayed using the feral mouse cells SC-1 (4) or NIH/3T3 (5) cells and xenotropic virus was titered on the mink lung cell line Mv-1-Lu (6).

Viral Assays. Three types of assays were used: the XC test (7), the DNA polymerase induction assay (4), and the fluorescent-focus induction test (4). For infection, cells were seeded at 1×10^5 per 60-mm dish. One day after seeding, the cells were treated with Polybrene (20 g/ml) for 1 hr and then infected with 0.2 ml of virus suspension. After incubation for 2 hr, the virus inoculum was removed and 5 ml of complete medium was added. Twenty-four hours later, cultures were processed for indirect immunofluorescence with fixed cells as previously described (4). Culture fluids were collected at early confluency for determination of particle-bound DNA polymerase activity (4); at this time cells were lethally UV-irradiated and overlayed with 1×10^6 rat XC cells per 60-mm dish. Parallel dishes were subcultured and tested for virus at subsequent passages. Virus cloning was performed either by isolation from large plaques that developed in cultures infected at limiting dilutions or by the microtiter technique (8).

Growth in Soft Agar. To test the growth potential of infected and control cells in soft agar, cells were seeded into 0.3% Noble agar made up in complete medium containing 10% fetal calf serum, and 2 ml was plated on top of 5 ml of 0.5% basal agar. The plates were fed with top agar once a week and the fraction of cells able to grow to colonies in soft agar was determined after 2 and 3 weeks of incubation at 37° by using a stereoscopic microscope.

Abbreviations: R, replicative function (for quantitation see Fig. 4); XC^+ or XC^-, ability or inability to form syncytial cell plaques on rat XC cells; NL, nonleukemogenic (no leukemias after 18 months); SL, slowly leukemogenic (leukemias developing later than 9 months after inoculation); RL, rapidly leukemogenic (leukemias developing in less than 4 months); MuLV, murine leukemia virus.

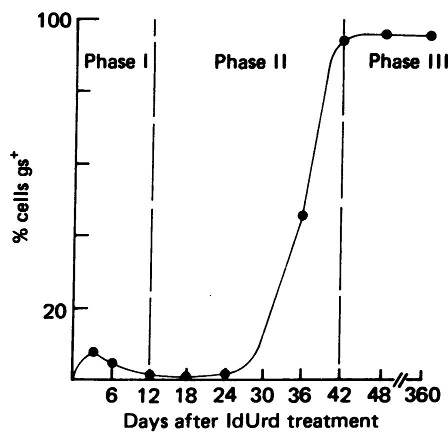

FIG. 1. Activation of endogenous type C virus from C3H/10T1/2 CL8 cells with IdUrd. Cells were treated at 50 μg/ml for 18 hr in the logarithmic phase of growth. Virus expression in treated cultures was monitored by indirect immunofluorescence for cytoplasmic group-specific (gs) antigens. Continued subculture of IdUrd-treated cells leads to slow spread of R^-XC^-NL murine leukemia virus (MuLV) in phase II and persistent infection in phase III.

RESULTS

Origin of the Nononcogenic Parent Virus. A virus was isolated from C3H/10T1/2 CL8 cells (1) after activation with IdUrd. As shown in Fig. 1, these cultures produce a replication-deficient (R^-) XC test negative (XC^-) virus that spreads slowly through the culture so that after six to eight subcultures (in phase III) over 90% of the cells are producing virus (3). The virus-producing culture was single cell cloned. One particular high-producer clone (clone A2) was the source of the R^-XC^- nontransforming, nonleukemogenic (NL) virus used in subsequent studies.

Derivation of High-Leukemia MuLVs in Cell Culture. Fig. 2 summarizes the two routes used to derive highly infectious MuLV. The first method involved selection for rapid replicating ability in transformed mouse cells in culture. The second method involved virus replication in thymus tissue *in vivo*.

The wild mouse cell line, SC-1, was infected with the R^-XC^- virus. The virus produced was serially transferred 8 to 12 times, yielding a virus able to grow to high titers in a variety of "N-type" mouse cells, including C3H/10T1/2 and NIH/3T3 (2). Similarly altered viruses have been produced by serially transferring the R^-XC^- virus in methylcholanthrene-transformed C3H/10T1/2 cells. The conversion to high-titer growth occurs in two discrete steps: the first leading from R^-XC^- to R^+XC^+ virus (4), and the second from R^+XC^+ to $R^{++}XC^+$ (Fig. 3). The resulting virus was cloned from large XC plaques and grew to very high titers on susceptible mouse cell lines. The pattern of conversion shown in Fig. 3 with SC-1 cells is similar to that with methylcholanthrene-transformed C3H/10T1/2 cells. The first conversion was observed after two to four virus subcultures and the second conversion occurred after three to eight additional transfers. Concomitant with, or shortly after, the first and second conversions, a xenotropic virus was transiently released; its time of appearance is indicated by the arrows in Fig. 3.

Derivation of High-Leukemia MuLVs *In Vivo*. MuLV was isolated from the thymus of NIH Swiss mice inoculated as newborns with either R^-XC^- or R^+XC^+ virus (see Fig. 2). The virus studied in detail was from the thymus of a 2-month-old symptom-free NIH Swiss mouse inoculated at birth with the poorly replicating R^-XC^- isolate. Mouse-tropic, xenotropic, and dual-tropic viruses were isolated from leukemic tissues.

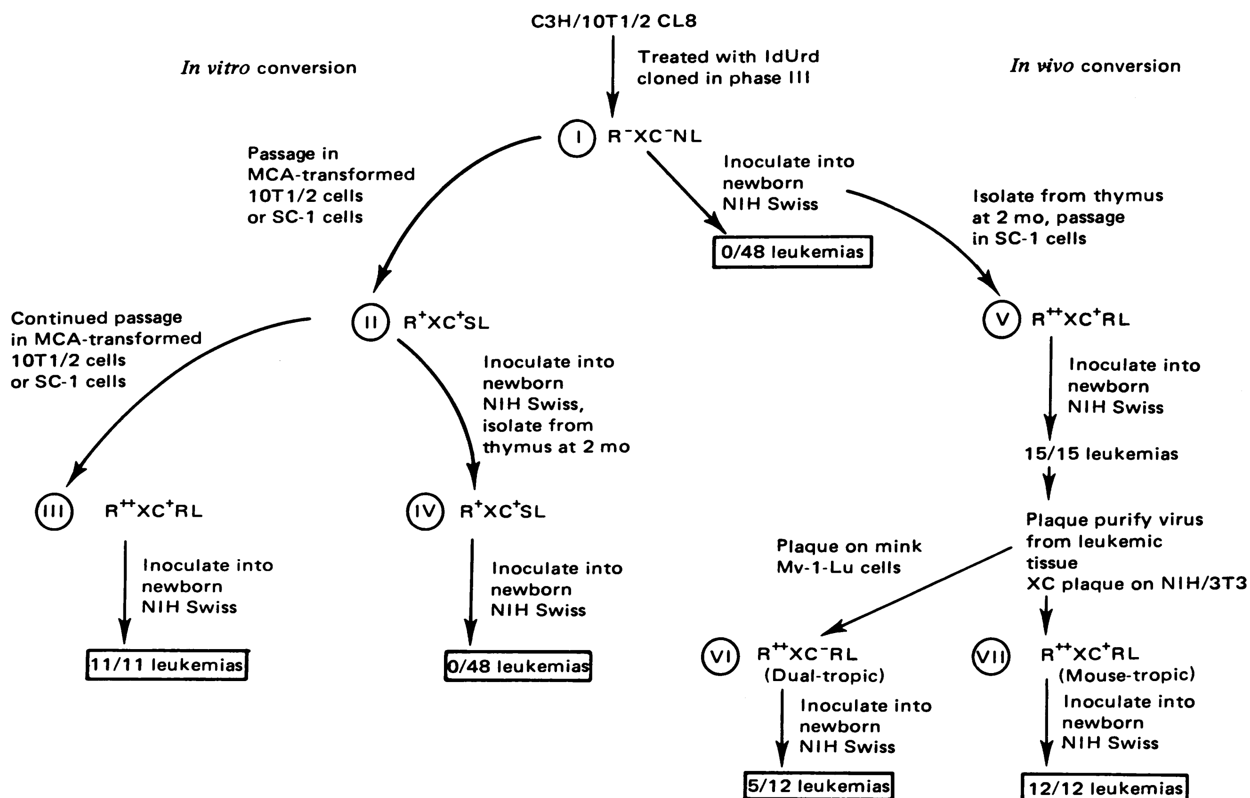

FIG. 2. Derivation of rapidly leukemogenic (RL) mouse type C viruses from R^-XC^-NL MuLV of C3H/He mice. MCA, methylcholanthrene; SL, slowly leukemogenic.

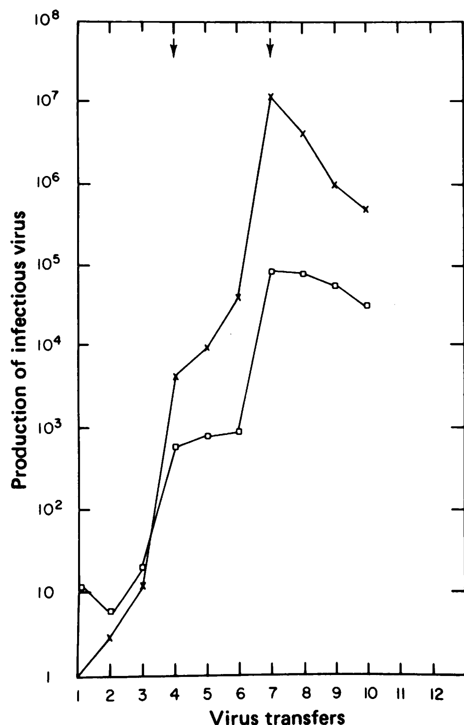

FIG. 3. Generation of rapidly transforming MuLV *in vitro*. Titer of infectious virus produced by cultures infected with successive virus transfer. □, PIU/10^5 infected cells; ×, XC plaque-forming units per 10^5 infected cells. One PIU is the amount of virus that leads to the production of RNA-dependent DNA polymerase (reverse transcriptase)-containing particles with an activity of 0.5 pmol of [^{3}H]-dTTP incorporated per 60 min in cultures infected according to previously described procedure (9). R^-XC^-NL is the virus used for the first infection. The arrows indicate the time of transient appearance of a xenotropic endogenous virus in the culture fluid of the infected cells.

Representatives of each of the three host range types were grown in mouse and/or mink cells (Fig. 2). It is not yet clear how much contribution the recipient animal may have made to the resultant viruses.

Host Range of MuLV Variants. Cloned viruses obtained as described above were grown in SC-1 or mink cells to determine their relative plating efficiencies (Table 1). Three host range classes of infectious MuLV were identified: mouse-tropic, xenotropic (growing on mink but not mouse cells), and dual-tropic. The viruses that grew to the highest titer on NIH/3T3 cells were XC^+ mouse-tropic or XC^- dual-tropic MuLV. Upon infection of mink cells, the latter viruses formed direct cytotoxic plaques as well as foci of morphologically altered cells similar to those described for viruses isolated from AKR mice (10, 11). The viruses were cloned either by isolation from cytotoxic plaques that developed at limiting dilutions or by endpoint cloning of polymerase-inducing virus on mink cells. The latter procedure was also used for the purification of xenotropic MuLV. The dual-tropic viruses were not phenotypically mixed, because they did not segregate pure mouse- or xenotropic virus through eight serial transfers in culture or following growth in NIH Swiss mice for several months.

Tumorigenicity of the Host Range Variants. Inocula (2–5 × 10^3 infectious units) were injected intraperitoneally into newborn NIH Swiss mice, which were monitored for the development of viremia and tumor formation. The N-tropic R^-XC^- virus from C3H produced viremia infrequently (2/48) and did not induce disease within 6 months. This virus is termed R^-XC^-NL (nonleukemogenic). The *in vitro* derived R^+XC^+ variant established early viremia and a productive infection in the thymus and other lymphoid tissues. Most of the animals developed lymphocytic leukemia later in life, between 9 and 18 months. The virus is slowly leukemogenic (SL). Both the *in vitro* and *in vivo* derived $R^{++}XC^+$ viruses caused lymphocytic leukemia in all inoculated mice with a mean latency period from 67 to 96 days (see Table 1). These viruses are thus designated $R^{++}XC^+RL$ (rapidly leukemogenic). The earliest leukemias were predominantly splenic; those developing later involved the thymus and lymph nodes. The dual-tropic MuLV clone, 64 PL clone 1, also induced rapid leukemia in newborn NIH Swiss mice but at a decreased incidence (41%). Lymphoid tissues revealed leukemic cells in the spleen as early as 3 weeks after inoculation with the latter virus.

Solid tumors detectable at autopsy developed at the injection site. Most of these were sarcomas originating in muscle, connective, or lymphoid tissue. Two of the four solid tumors in the animals inoculated with R^+XC^+SL, however, were ovarian carcinomas developing between 3 and 4 months (Table 1).

Spreading Rate of Variant MuLVs in Culture. Rapidly transforming type C viruses can be derived in cell culture and in the animal. Isolation does not require passage *in vivo* through thymus cells but can be accomplished entirely in cell culture. What are the changes that occur in forming the rapid leukemia viruses? Because both the mouse-tropic and dual-tropic viruses induce leukemias rapidly, the acquisition of the ability to grow in or to transform mink cells is not a ***necessary*** feature of increased leukemogenicity. Pathogenicity, however, correlates with the ability to grow rapidly in mouse cells. Fig. 4 shows the results of a representative experiment in which 10T1/2 cells were infected and virus growth was followed daily by determination of DNA polymerase-containing particles. The viruses fit into three groups on the basis of the slope of their spreading curves: (*i*) Poorly infectious mouse-tropic virus, the prototype being the endogenous R^-XC^-NL virus from C3H or BALB/c. This virus requires several weeks to reach maximum growth.

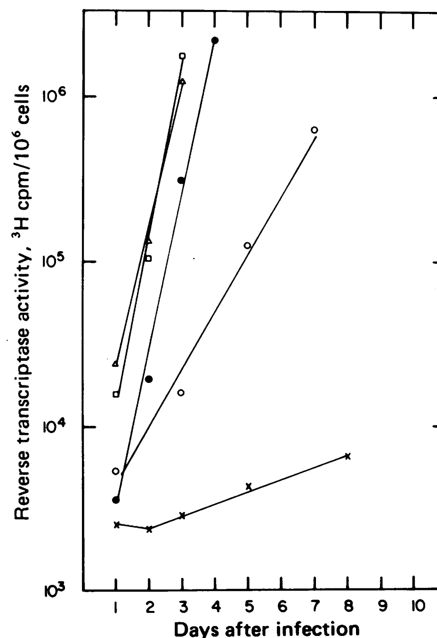

FIG. 4. Growth of variants derived from R^-XC^-NL MuLV. C3H/10T1/2 CL8 mouse cells were infected at multiplicities of infection of 0.1–0.3 and particle-bound reverse transcriptase was determined in culture fluids of infected cells at daily intervals. ×, R^-XC^-NL; ○, R^+XC^+SL *in vitro*; ●, $R^{++}XC^+RL$ *in vitro*; □, NIH PL CL6; △, 64 PL CL11.

Table 1. Host range, replicating ability, and leukemogenic potential of variants derived from R^-XC^- virus of C3H mouse cells

			Growth in test cell culture				Tumorigenicity in newborn mice			
			Mouse NIH/3T3		Mink Mv-1-Lu		Fraction of mice diseased within 6 mo		Mean latency period, days	
Virus	Designation (from Fig. 2)	Host range class	PIU/ml	XC PFU/ml	PIU/ml	Mink PFU/ml	Solid tumors	Leukemias	Solid tumors	Leukemias
R^-XC^-NL	I	Mouse	3.4×10^4*	<5	0.5	<5	0/48	0/48	NA	NA
R^+XC^+SL (*in vitro*)	II	Mouse	1.2×10^4	9.3×10^4	0.5	<5	4/48†	0/48	~60–180	NA
$R^{++}XC^+RL$ (*in vitro*)	III	Mouse	**8.2×10^7**	2.1×10^8‡	1.8×10^1	<5	2/11	**11/11**	~60	96
R^+XC^+SL (*in vivo*)	IV	Mouse	6.5×10^4	8.6×10^4	0.5	<5	0/13§	0/48	NA	NA
$R^{++}XC^+RL$ (*in vivo*)	V	Mouse	**1.3×10^7**	3.5×10^7	3.0×10^1	<5	1/15	**15/15**	~60	83
$R^{++}XC^-$ clone 11	VI	Dual	**7.0×10^6**	<5	4.3×10^6	3.5×10^6	0/9	**5/12**	NA	105
NIH Xeno clone 1	—	Xeno-	<0.5	<5	1.1×10^5	<5	0/12	0/12	NA	NA
$R^{++}XC^+$ clone 6	VII	Mouse	**4.5×10^7**	1.1×10^8‡	2.2×10^1	<5	2/12	**12/12**	~60	67

PIU is defined in the legend to Fig. 3. PFU, plaque-forming unit; NA, not applicable. Numbers in **bold-face** indicate high plating efficiencies and leukemogenicities.

* Virus growth for the R^-XC^- stock was titered by the fluorescent focus assay.

† Two of the four tumors were ovarian carcinomas.

‡ This virus stock also forms direct syncytial plaques on various mouse cells in the absence of added XC cells.

§ Only 13 of the 48 animals in this series were completely autopsied.

The final titers produced were 10^3–10^4 infectious units. (*ii*) Infectious, XC^+ mouse-tropic virus, the prototype being the R^+XC^+SL virus derived from R^-XC^-NL *in vitro*. The rate of spread, as well as the lag period after infection and the final titers produced, are similar to the values obtained in the first group. N-tropic AKR MuLV were isolated from tail extracts of young adult mice (12). (*iii*) The *in vitro* and *in vivo* derived $R^{++}XC^+RL$ mouse-tropic and $R^{++}XC^-RL$ dual-tropic variants, which have a very high spreading rate and achieve final titers 10^2–10^3 times greater than those obtained with the R^+XC^+SL viruses.

Growth of Virus-Infected Cells in Soft Agar. Several observations have been made which show that the R^{++} viruses affect the cell growth properties. The dual-tropic viruses ($R^{++}XC^-RL$) form cytotoxic plaques on mink cells. Foci of altered growth are also seen, although overgrowth of the cell monolayer is less pronounced than that caused by laboratory strains of sarcoma viruses. Morphologically altered cells are often seen at the edges of cytotoxic plaques. Fresh isolates of uncloned $R^{++}XC^+RL$ virus from the leukemic animal showed plaque formation in NIH/3T3 cells as a linear function of dose. These plaques contained syncytial cells, developing 2–3 days after infection, associated with areas of overgrowth and rounding of cells. Focal areas made up of cells with the latter characteristics were also seen in the absence of direct plaque formation. Upon continued passage of the virus the cytotoxic effect diminished and the ability to induce focal areas was lost. Similar morphological alterations have been observed with pathogenic variants of avian leukosis viruses (13).

The experiment described in Fig. 5 was performed to quantitate the transforming ability of these rapidly leukemogenic variants. C3H/10T1/2 CL8 cells were infected and seeded into soft agar. Colony growth was monitored for 3 weeks. Infection with cloned viruses of the R^+XC^-sl or $R^{++}XC^+RL$ group resulted in an increase in the fraction of cells growing in agar, and in the size of the colonies (Fig. 5). Infection with Moloney murine sarcoma virus with MuLV coat [MSV (MuLV)] induces a still larger fraction of cells to grow to even larger colony size. This experiment shows that infection with the leukemia-inducing viruses induces a fraction of the cells to anchorage-independent growth, a property commonly associated with tumorigenicity of cells *in vivo* (14). The ability to

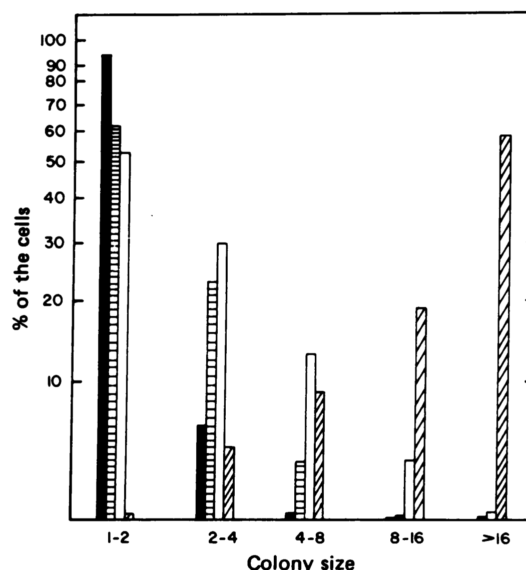

FIG. 5. C3H/10T1/2 CL8 cells were infected at multiplicities of infection >3 seeded into 0.3% soft agar plated on top of 5 ml of 0.5% agar in 60-mm dishes. Colonies developed within 2–3 weeks after seeding. A variety of colony sizes was observed. The percentages of the cells found in colonies of various sizes are shown; sizes are indicated as multiples of the size of single cells. Solid bar, uninfected control cells; horizontally hatched bar, cells infected with R^+XC^+SL virus; open bar, cells infected with $R^{++}XC^+RL$ virus; obliquely hatched bars, cells infected with Moloney murine sarcoma virus with MuLV coat.

induce growth in agar parallels the ability to produce leukemias and solid tumors *in vivo*.

DISCUSSION

This report describes the isolation of highly leukemogenic type C viruses from poorly infectious, nononcogenic, endogenous mouse type C viruses. Repeated transfer of virus in transformed mouse fibroblast cell lines led to formation of high-titered, rapidly leukemogenic mouse-tropic MuLV, activation of xenotropic MuLV, and the appearance of viruses with a dual host range. In the animal the process was also accompanied by activation of xenotropic MuLV. Inoculation of the high-replicating mouse-tropic MuLV into newborn NIH Swiss mice led to the development of lymphocytic leukemia within 4 months in 100% of the animals. A comparison of the leukemogenicity of the three different host range classes of viruses showed that the $R^{++}XC^{+}RL$ mouse-tropic viruses were the most pathogenic isolates, followed by the dual-tropic MuLV.

The most characteristic properties common to the highly oncogenic variants are their rapid and high-titer growth *in vivo* and *in vitro* and their ability to induce growth of fibroblasts in soft agar. The xenotropic clonal isolates, in contrast, could not be demonstrated to be tumorigenic in newborn NIH Swiss mice. The mouse-tropic $R^{++}XC^{+}RL$ viruses also induce solid tumors *in vivo* and anchorage-independent growth of infected cells *in vitro* at low frequency. The mouse-tropic XC^{+} isolates and dual-tropic XC^{-} isolates are rapid leukemia inducers. They are also the most efficient spreaders, transform cells in culture, and produce solid tumors in the animals. The solid tumors include carcinomas and sarcomas of various degrees of differentiation.

Whatever the mechanisms by which rapidly leukemogenic viruses are generated from nonleukemogenic variants, the results show that the ability of mouse-tropic MuLV to induce tumor formation *in vivo* correlates directly with its replication efficiency and spreading rate. We have also shown that highly virulent MuLV can be derived *in vitro* from endogeneous nononcogenic type C virus by selecting for high-titered growth in fibroblasts. Similar events may occur during development of spontaneous leukemia *in vivo*. This seems likely in view of earlier reports describing the isolation of a leukemogenic MuLV from lymphoreticular tumors of inbred BALB/c mice while the mouse-tropic virus produced by young mice of this strain has not been demonstrated to be oncogenic in the natural host (15).

Type C viruses have been isolated from preleukemic tissues of inbred AKR mice, a high-leukemic strain, that differ from the mouse-tropic MuLV found in young animals from this strain. This led to the identification of a new class of dual-tropic leukemia-inducing type C viruses designated MCF (10). The viruses contain components that grow in mouse and mink cells and form foci on mink cells (11). They are found in preleukemic thymuses from high-leukemic strains and, therefore, are implicated in disease development (10). Evidence has been presented that they form as a result of recombination between mouse-tropic and xenotropic endogenous viruses (10, 11). The dual-tropic, mink cell-transforming, XC^{-} viruses described here have many properties similar to those of the MCF isolates. They can, however, be obtained entirely from tissue culture cells of low-leukemia mouse strains. The dual-tropic viruses described here have a viral envelope glycoprotein, gp70, very much like the MCF viruses by serologic tests, but the properties of the small internal viral proteins (p12 and p15) are distinctly different. Dual-tropic plaque isolates differ from one another and from the MCF viruses in the immunologic properties of their small proteins (unpublished experiments).

The observations described in this report suggest a strategy for the isolation of new rapidly transforming viruses. The first step is the adaptation of a given virus to high-titer growth. This leads to the emergence of virus with some, albeit limited, potential for fibroblast transformation, inducing morphological alterations and growth in soft agar *in vitro*. Because agar colonies usually do not grow progressively, the transforming effect of these viruses may initially be more akin to the "abortive transformation" effect described for the DNA tumor viruses (16). Through further selection of virus from such focal areas of altered growth, more efficiently transforming viruses may be isolated and characterized.

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