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Non-thymic Malignant Lymphomas Induced in C57BL/6 Mice by Cloned Dualtropic Viruses Isolated from Hematopoietic Stromal Cell Lines*

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Abstract—*Retroviruses cultivated from adherent bone marrow cells of C57BL/6 mice have been cloned* in vitro *and were shown to be potent inducers of reticulum cell neoplasms (RCN) (a* non-thymic *malignant lymphoma) in C57BL/6 mice. The RCN-inducing isolates were high-titer dualtropic viruses and presumably were recombinant in the major envelope glycoprotein gp70. The virus clones constituted part of a larger group of equally high titer dualtropic isolates most of which were innocuous* in vivo. *Previously, we have shown that* thymic *malignant lymphomas were induced by other, thymotropic viruses isolated from C57BL/6 mice, viruses that were shown to be recombinant in gp70. Thus, non-thymic malignant lymphomas were induced in C57BL/6 mice by specific dualtropic isolates, whereas thymic malignant lymphomas were caused by other, distinctive recombinant viruses.*

INTRODUCTION

A UNIQUE class of murine leukemia viruses (MuLVs) associated with the spontaneous appearance of thymic lymphomas in AKR mice have been isolated from preleukemic and leukemic thymic tissue [1]. These newly found viruses induced cytopathic changes in mink lung fibroblasts and were, therefore, called mink cell focus (MCF)-inducing viruses. MCF viruses are dualtropic. That is, their envelope glycoproteins (gp70s) are intragenic recombinants, with portions of their gp70s derived from both ecotropic and murine xenotropic parental viruses [2]. MCF viruses have also been isolated from the Moloney MuLV complex [3, 4], and from the Friend erythroleukemia-inducing virus complex [5]. Moreover, leukemogenic thymotropic radiation leukemia virus from C57BL/6 (B6) mice [6] also has a recombinant gp70 [7].

In a preceding paper [8], we reported the cultivation of reticulum cell neoplasm (RCN)-inducing MuLV in stromal cell lines of hematopoietic organs, derived from B6 mice bearing RCNs. Virus harvested from these stromal cell lines induced splenic and lymph node RCNs in inoculated B6 mice within 30 days. Furthermore, both mouse and mink cells were susceptible to infection with these potent RCN-inducing viruses and produced high titers of infectious virus [8]. However, it was not clear whether these two infectious activities were originated from two or more separable classes of virus particles, in dualtropic particles or both. Nor was it obvious whether dualtropic or ecotropic viruses harbored RCN-inducing capacity. Hence, we set out to clone viruses from the apparent virus mixture by growing out progeny of single virus infectious particles in SC-1 mouse fibroblasts, in mink lung cells, and in NTE cells, a stromal epi-

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Abbreviations: MCF, mink cell focus-inducing; MuLV, murine leukemia virus; B6, C57BL/6 mice; RCN virus, reticulum cell neoplasm virus; FA, fluorescent antibody; m.o.i., average multiplicity of infection; i.p., intraperitoneal inoculation; CPE, cytopathic effect; NTE, normal thymus epithelial cell line; MLC, mink lung cells.

thelial cell line from a B6 mouse which we used because virus originally inhabited and grew particularly well in stromal hematopoietic cells [8]. Viruses were cloned separately in the three cell lines for we anticipated that the biologically active RCN virus might be lost upon cloning in some, though not in other cells.

We have now derived from an RCN stromal cell line a series of viruses by endpoint dilution cloning in mink and mouse cells. Most of the viruses cloned in this way were dualtropic and presumably recombinant in gp70; several were ecotropic. One-third of the dualtropic isolates were potent inducers of MCF *in vitro*, and several induced RCN *in vivo*.

Thus, some cloned dualtropic virus isolates were potent inducers of RCN, though *in vivo* RCN tumor-induction and *in vitro* mink cell focus (MCF)-induction appeared to be separate properties of these dualtropic viruses.

MATERIALS AND METHODS

The viruses to be cloned and isolated were derived from the stromal fibroblastoid cell line RCN-BM5 grown from the bone marrow of a B6 mouse carrying an RCN [8]. The source of non-cloned virus and the RCN tumors it produces have been described [8]. All virus infections were assayed and titrated by the fluorescent antibody (FA) procedure as described [8]. In short, cells that were growing on glass slides were infected with dilutions of virus (as 0.2 μm-filtered 24-hr culture supernatant fluids). Seventy-two hr post infection the slides were fixed in acetone and centers of virus replication were counted following FA-staining of the slides using a broadly reactive rat anti-MuLV serum and a FITC-conjugated anti rat IgG serum. RCN viruses were cloned separately on SC-1 mouse cells, on mink lung cells, and on the NTE cell line (a cell line derived from the thymic epithelium of a 6-week-old B6 mouse).

Virus was cloned on five groups of 48 separate cell cultures, each infected with a different four-fold dilution of filtered virus from RCN-BM5 supernatant (dilutions 5×10^3 to 5×10^6). Infected cultures were subcultured three times to allow viral spread. Only cultures that were infected at virus dilutions yielding, at most, six FA-positive (infected) cultures out of 48 (m.o.i. $\simeq 0.1$) were used in this study. Such virus isolates were considered to be the progeny of a single infecting virus particle. Under these conditions the probability of a culture becoming doubly-infected is less than 1 in 100 [9]. Some viruses were recloned by the same procedure. In total, 18 cloned isolates were studied. The six isolates cloned on SC-1 cells were designated S(3---), those cloned on mink cells as M, and those cloned on NTE cells as N.

N/B ecotropism was tested on the Fv-1 congenic cell lines SIM/SIM.R, [10], and was assayed by the FA procedure [8]. The titer of B-tropic virus was 300–2000-fold higher on SIM.R ($Fv\text{-}1^{bb}$) than on SIM ($Fv\text{-}1^{nn}$) cells.

Virus neutralization and interference were assayed by the FA procedure. The viral isolates were evaluated for tumor inductive capacity *in vivo* by i.p. inoculation of 0.1 or 1.0 ml of filtered culture supernatants in groups of 12 young adult B6 mice. Inoculated mice were checked twice weekly for palpable tumors during at least 6 months. After sacrifice, all tumors were examined histologically. Virus infections were always done following filtration through 0.22 μm filters and contained 16 μg/ml polybrene for the first 4 hr.

RESULTS

Dualtropic viruses cloned from stromal RCN bone marrow cell line

The host range and infectious titer of the viruses cloned on three cell lines shows that 16 of 18 isolates were dualtropic, high titer viruses (Table 1). The isolates that were cloned on mink cells consistently displayed a higher infectious titer on mink than on mouse cells; similarly, isolates cloned on mouse cells grew somewhat higher titer on mouse cells. Two of the six isolates cloned on SC-1 cells proved to be ecotropic viruses. All isolated clones were B tropic on mouse cells. Isolation of these cloned viruses from RCN-BM5 cells did not involve any selective procedures, because the assay used throughout, FA, detects replicating MuLVs of all classes. Thus, no preference was given to the isolation of dualtropic virus. Nevertheless, of the 12 clones selected on mouse cells, 10 were dualtropic (Table 1). This indicated that titer of infectious dualtropic virus in the RCN-BM5 cell line was six-fold higher than that of ecotropic virus. No xenotropic virus was found among the six mink cell-grown isolates. When several of the viruses listed in Table 1 were recloned twice by endpoint dilution, no change in their properties was apparent (not shown).

Cytopathic effect (CPE) induction in mink cells by cloned RCN virus isolates

All six virus clones that were isolated on mink cells induced CPE in mink cells (Table 2). Only two of the six dualtropic viruses cloned on NTE cells and two out of four dualtropic clones isolated on SC-1 cells induced CPE in mink cells, although all the mouse-cell-isolated dualtropic viruses infected mink cells with high titers. Among isolates inducing CPE in mink cells, distinct variations of their cytopathic behavior were evident. Some viruses continuously killed 99% or more of the chronically infected mink cells. Others retarded mink cell growth through continuous cell killing, so that the infected cultures never reached confluency. A third manifestation of the mink cell cytopathic isolates was the initiation of floating clusters of rounded, live cells as well as foci of dead cells (Fig. 1). It is not clear whether the clusters of virus-infected cells were transformed mink cells. It has been difficult to titrate both the mink cell cluster-inducing viruses and the MCF inducing viruses. However, one can easily quantitate the virus-induced retardation of mink cell replication by replating the virus infected mink cells and comparing growth rates in the two cultures (not shown).

Neutralization of dualtropic RCN viruses

The dualtropic virus isolates were tested for their sensitivity to neutralization by antisera to ecotropic envelope glycoprotein gp70 and to xenotropic (NZB) purified virus. Ecotropic clones that were co-isolated with the dualtropic RCN isolates served as convenient internal controls. Table 3 shows representative neutralization tests of dualtropic isolates assayed on SC-1 and mink cells, and the variability of neutralization. The values for the neutralization among viruses within the group of dualtropic isolates varied considerably more than the neutralization values obtained by assaying any one isolate both on SC-1 and on mink cells (one example shown in Table 3).

Table. 1. *Host range (expressed as infectious titer) of RCN-virus clones**

Cells used for virus cloning	No. of clones with virus cloning	Indicated tropism	Range of infectious titer	
Mink	6/6	D;B‡	$2–4 \times 10^5$	$1–2 \times 10^5$
NTE§	6/6	D;B	$2–4 \times 10^4$	$2–4 \times 10^5$
SC-1	4/6	D;B	$1–2 \times 10^5$	$2–3 \times 10^5$
	2/6	E;B	0	$2–3 \times 10^5$

*Virus was cloned by infecting multiple cultures of the three cell lines indicated with high dilutions of supernatant virus from stromal bone marrow cell line RCN-BM5 [8]. Clones were picked from those cultures that had been infected at a m.o.i. of less than 0.1 virus particles/culture, as verified statistically.

†N/B ecotropism was tested on the Fv-1 congenic cell lines SIM/SIM.R, data not shown.

‡D, dualtropic; E, ecotropic; B, B-tropic

§Normal thymus epithelial cell line grown from young adult B6 thymus reticulum.

Table 2. *Cytopathic effect (CPE) induced in mink cells by dualtropic RCN virus isolates**

Cells on which clones were isolated	No. of clones inducing CPE/ No. dualtropic clones isolated	No. of clones inducing RCN *in vivo*/ No. dualtropic clones isolated
Mink	6/6	1/6†
NTE	2/6	1/6‡
SC-1	2/4	0/4

*High titer cloned virus was used to infect mink lung cell cultures. Infection was monitored by the FA procedure. CPE was sought 3–5 days after infection, or following subculturing the confluent cells at a ratio 1:20. See text and Fig. 1 for variable manifestations of mink cell CPE that was induced by the various virus clones.

†Clone M(BC4) was a potent inducer of mink cell CPE.

‡Clone N(GB3) did not induce CPE in mink cells.

Table 3. Representative neutralization tests of dualtropic RCN-viral isolates and ecotropic and xenotropic MuLVs

Virus			Cells infected in neutralization test	Virus neutralization by antisera*			
				Rabbit anti-AKR gp70 ecotropic virus	Goat anti-NZB purified xenotropic virus	Normal serum	
Strain	Tropism	Propagated in				Rabbit	Goat
N(GB6)	D	NTE	SC-1	+	++	−−	−−
N(GB5)	D	NTE	SC-1	++	+++	−−	−−
M(BA1)	D	Mink	SC-1	++	+++	−−	−−
M(BC4)	D	Mink	Mink	++	+++	−−	−−
S(FC6)	D	SC-1	SC-1	+++	+++	−−	−−
			Mink	++	+++	−−	−−
S(FA2)	B	SC-1	SC-1	+++	−−	−−	−−
Control viruses							
1A4	B	SC-1	SC-1	+++	−−	−−	−−
54-1-5X	X	Mink	Mink	−−	++	−−	−−

*Virus was neutralized by incubation for 1 hr at room temperature with the antisera or the normal sera at a final dilution of 1:100 for rabbit sera and 1:20 for goat sera. Virus infectivity (at dilutions 1:1; 1:5; 1:10) was titrated by the FA procedure. −− = ≦20% reduction of infectious titer, +++ = ≦95% neutralization of infection; ++ =60–95% neutralization; + =40–60% neutralization. The anti-xenotropic serum made in goats was kindly supplied by Dr. Fred Jensen.

Interference of dualtropic isolates by ecotropic and xenotropic MuLVs

In this paper we used a novel two-step virus interference test (suggested to us by Dr. Fred Jensen). The test took advantage of the dualtropic nature of the virus isolates. Interference tests of dualtropic virus isolates by ecotropic and xenotropic viruses were carried out as follows. First, cultures of mink lung cells (MLC) and MLC chronically infected with xenotropic virus (MLC-X) were infected with the dualtropic isolates. In a second infection step supernatants from step 1 were used to infect SC-1 cells. The extent of infection in the second step (and, thus, the degree of interference in the first step) was followed by the FA procedure and by assaying culture supernatants for reverse transcriptase. A corresponding but opposite procedure was followed to test for ecotropic virus interference. Xenotropic virus strongly interfered with the infectivity of all dualtropic isolates (Table 4). The degree of interference by xenotropic virus was so strong that upon repeated subculture of the second step SC-1 cutures only a few produced infective dualtropic virus. Interference by ecotropic virus (B tropic virus isolate 1A4) was more variable, ranging from 40 to 95%. Interference by ecotropic virus of dualtropic isolates cloned in mouse cells was consistently more pronounced (80–95%) than that of isolates cloned in mink cells (40–60%).

In vivo *induction of non-thymic lymphomas (RCN) by cloned dualtropic isolates.*

Of the 16 dualtropic viral isolates cloned from the stromal cell line RCN-BM5, two induced RCN upon i.p. inoculation into B6 mice. None of the other 16 viruses (two ecotropic, 14 dualtropic) induced any disease in three separate experiments (Table 2) (observation time 6 months). One isolate cloned on mink lung cells designated M(BC4) induced palpable RCN in 12 mice out of 12 inoculated in 51 days (9/12 in 39 days); the other biologically active isolate N(GB3) was grown on NTE and induced 8 RCNs in a group of 12 injected mice at 64 days. None of the other isolates, whether dualtropic (14 isolates) or ecotropic (two isolates) produced RCNs nor other malignancies, in adult or in newborn mice, even though the viruses grew to high titers *in vitro* and *in vivo* (data not shown).

Both the M(BC4) and N(GB3) isolates stably induced RCN *in vivo* whether grown in culture for 1 month or continuously for 12 months. Likewise, the innocuous dualtropic isolates were stable in culture for more than 12 months.

DISCUSSION

Virus clones that induced non-thymic lymphomas (RCNs) *in vivo* were isolated from the stromal, non-cloned bone marrow cell line RCN-BM5. The properties of these viruses

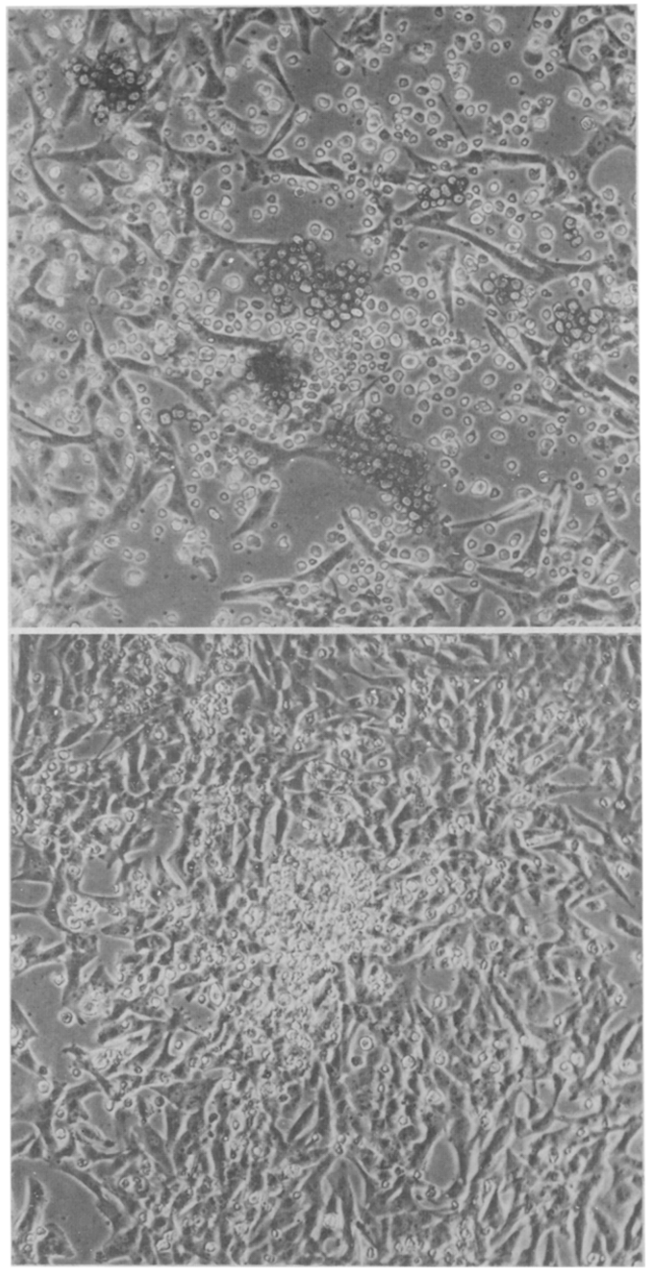

Fig. 1. *CPE induced in mink lung cells by RCN viruses cloned on mink cells. Top: mink cell focus of dead cells induced by the M(BB1) virus clone. Bottom: mink cell clusters consisting of colonies of live suspension cells induced by the M(BA4) virus clone* (×330).

Table 4. Interference of dualtropic RCN viral isolates by ecotropic and xenotropic MuLVs

Virus isolate†	Infectious virus after passage through cells pre-infected with interfering virus* 1st infection: / 2nd infection:				
		MLC-X / SC-1	MLC / SC-1	SC-1-E / MLC	SC-1 / MLC
N(GB6)		0‡	100	5§	100
N(GB5)		0	100	20	100
M(BA1)		0	100	60	100
M(BA4)		0	100	40	100
S(FC6)		0	100	10	100
S(FB1)		0	100	15	100

*Viral interference was tested by (i) infecting MLC and xenotropic virus-infected MLC (MLC-X), as well as SC-1 cells, and ecotropic virus-infected SC-1 cells (SC-1-E; B-tropic clone 1A4) with undiluted dualtropic RCN viral isolates. Medium was changed daily. Ninety-six hr after the first infection (ii) new SC-1 cultures were infected with undiluted supernatants of the (step I) infected MLC cultures, and new MLC cultures were infected with supernatants of the (step I) infected SC-1 cultures. Ninety-six hr later the second cycle of infection was assayed by the FA procedure on fixed cells growing on slides. The percentage of FA positive cells was recorded. Results were double checked by assaying for reverse transcriptase activity in supernatants of the (step II) infected cultures (not shown).

†Experiments with ecotropic and xenotropic viruses and with uninfected controls were omitted from this table.

‡§Ratio of virus infectivity (%) remaining after first passing the virus through a pre-infected (interfering) culture compared to passing it first through uninfected MLC or SC-1 cells, respectively.

suggested that they were envelope glycoprotein (gp70) gene recombinants. This interpretation was based on the dual host range of the isolates, on their neutralization by antisera to ecotropic and xenotropic viruses, and on the interference of infectivity by ecotropic and xenotropic viruses. Thus, like the thymic lymphoma-inducing viruses of C57BL/6 mice [7], RCN-inducing viruses may also be unique recombinant species. Moreover, MCF-inducing viruses that are associated with induction of lymphosarcomas in AKR mice [1] and erythroleukemia inducing Friend virus isolates [5] have been shown to be recombinants in the envelope glycoprotein gp70 [2]. These RCN-inducing viruses are only two clones from a group of 18 viruses isolated from the bone marrow-derived RCN-BM5 cells. Each of the 18 clones was isolated as the progeny of single infectious virus particles, 16 of which turned out to be dualtropic. Eighty-eight per cent (88%) of these dualtropic isolates (14 of 16) did *not* induce disease, suggesting that within this group of isolated dualtropic viruses only a small percentage were accorded the specific disease-inducing determinants.

What specific viral characteristic endows a dualtropic virus with the ability to induce RCN *in vivo*? One possible trait would be the presumed recombinant feature of the viral gene coding for gp70 [2]. However, *thymic* lymphoma-inducing virus from C57BL/6 mice also displays a recombinant gp70 [7], whereas the majority of cloned dualtropic 'RCN' viruses isolated by us did not induce any disease *in vivo*. Thus, whereas the ability to induce RCN *in vivo* may indeed reside in an altered gp70, the presumed recombinant gp70 that is associated with RCN induction should be a highly specific one. This assumption gains support from our finding that viruses of the group of dualtropic isolates described in this communication behaved heterogenously with respect to all the properties studied; host range, neutralization by antisera, interference, CPE in mink cells and induction of neoplasms *in vivo*.

One possible explanation for the lack of RCN-induction by 14 out of 16 isolated dualtropic viruses is that tumor cells induced by these viruses are immunogenic and, thus, never overtly expressed. This explanation seems improbable since immunosuppression of the recipient mice by 400 rad X-rays did not lead to tumor induction nor did virus inoculation into newborn B6 mice.

An alternative explanation for the RCN-inductive capacity of only two out of 16 dualtropic isolates is that a gene product not related to gp70 may determine the viral tumorigenic properties. Moreover, in spite of careful endpoint dilution cloning, the RCN-inducing isolates may harbor an undetected defective genome, which could be responsible for tumorigenesis. If a presumed defective genome were present at a titer similar to that of the dualtropic nondefective virus, it would go undetected through repeated endpoint dilution cloning procedures. However, one would expect to detect its present in specific molecular experiments. Pending such experiments, we cannot exclude the presence of such a replication defective agent in our RCN-inducing cloned isolates.

B6 mice bearing virus-induced nonthymic lymphomas do not suffer from viremia. Nevertheless, stromal cells grown from their hematopoietic organs harbor ample MuLV [8]; each of 31 stromal cell lines which we have established from hematopoietic organs produced potent RCN-inducing virus [8]. Furthermore, we have shown that following virus cloning, 16 out of 18 viruses (89%) were of the dualtropic type, suggesting that the stromal cells of hematopoietic organs are a natural habitat for dualtropic viruses. Therefore, one may justifiably surmise that the hematopoietic stromal cells are the site at which retrovirus recombination is high, and where some of the presumed recombinant viruses have a selective advantage in replicating and infecting bone marrow-derived target cells. A similar mechanism for the genesis of retrovirus-induced thymic lymphomas has been previously proposed [7].

The group of dualtropic viruses which we have cloned from the stromal bone marrow cells RCN-BM5 may represent the heterogeneous genesis of recombinants in such hematopoietic cells. However, we cannot now rigorously exclude the possibility that such heterogeneity may be the result of replication and isolation *in vitro* of the virus, whether in the stromal RCN-BM5 cells, or during virus cloning. Study of a group of recloned virus isolates should give an estimate of the degree of change occurring in dualtropic viruses in culture, after which one might decide whether the observed virus heterogeneity occurred at the source (*in vivo*) or in culture.

Isolation of dualtropic virus clones in mouse and/or mink cells without selecting for the MCF-inducing property gave rise to a heterogeneous group of viruses. We have shown that the 16 dualtropic viruses described in this communication differed with respect to the following properties: (i) the relative infectious titers on mouse and mink cells; (ii) neutralization; (iii) interference; (iv) RCN-induction *in vivo*; and (v) induction of CPE in mink cells. Whereas all six isolates that were cloned on mink cells were cytopathic to various degrees in other mink cell cultures, only four out of ten dualtropic clones isolated on mouse cells induced CPE in mink cells.

CPE induction in mink cells did not correlate with RCN-induction *in vivo* . One of the two RCN-inducing isolates was strongly cytopathic for mink cells [isolate M(BC4)], whereas the other [N(GB3)] induced no CPE in mink cells. The capacity to induce CPE in mink cells is a property of many dualtropic recombinant isolates [1]. However, this feature was dissociable from the potential to induce RCNs *in vivo*.

One property was shared by all of the 16 dualtropic isolates; that is, the failure to induce XC-plaques (when growing in SC-1 cells), although the two ecotropic isolates served as convenient internal controls in many of our experiments.

None of the cloned dualtropic viruses described here has been observed to induce thymic lymphomas, as some other dualtropic viruses do [11]. Nor have any of a larger number of MuLVs grown in stromal hematopoietic cells ever induced anything but RCN [8]. Thus, one might speculate that only highly specific intragenic gp70 recombinants induce RCN or thymic lymphosarcoma, respectively. Quite possibly, the RCN and thymic lymphoma-inducing viruses are only two of a large group of innocuous recombinant viruses that develop after X-irradiation of B6 mice. The possibility is supported by the present isolation of a polymorphic group of dualtropics, only two of which induced RCN, and by our observations that the RCN- and thymic lymphoma inducing viruses are each distinct with respect to their biological properties [8]. The present set of cloned viruses derived from RCN-stromal cells together with thymic lymphoma-inducing viruses that were

isolated earlier may enable us to compare the biochemical features of disease-inducing viruses with those of the innocuous viruses.

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