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Biological and serological characterization of radiation leukemia virus

(murine ecotropic and xenotropic endogenous C-type viruses/differentiation-specific host cell restriction/selective neutralization/radioimmune competition)

ALAIN DECLÈVE*, MIRIAM LIEBERMAN*, JAMES N. IHLE†, AND HENRY S. KAPLAN*

*Cancer Biology Research Laboratory, Department of Radiology, Stanford University Medical Center, Stanford, California 94305; and † NCI Frederick Cancer Research Center, Frederick, Maryland 21701

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ABSTRACT Radiation leukemia virus, isolated from radiation-induced lymphomas in C57BL/Ka mice and propagated in that strain, is thymotropic and leukemogenic *in vivo* but replicates poorly, if at all, in mouse and mink fibroblast cultures *in vitro*. Comparative studies indicate that this naturally occurring virus is distinct from the previously recognized classes of endogenous murine ecotropic and xenotropic C-type viruses which are capable of replication on fibroblasts (fibrotropic) but are neither thymotropic nor leukemogenic. These studies also demonstrate that a differentiation-specific restriction system governing the replication of the murine ecotropic C-type viruses operates in addition to the previously defined Fv-1 and SRV gene restriction systems.

Radiation leukemia virus (RadLV), a naturally occurring murine leukemogenic virus, is recovered from thymic lymphomas induced by x-irradiation in strain C57BL/Ka mice (1, 2). This virus replicates selectively in the host thymus (3, 4). Mice of strain C57BL have also been shown to harbor three classes of endogenous C-type viruses, all of which can be propagated on fibroblastic cells *in vitro* but differ in host range. They include B-tropic (5, 6), N-tropic (6, 7), and X-tropic viruses (8, 9). It is now reported that the thymotropic, leukemogenic particles in RadLV differ at the molecular level, as revealed by their antigenic determinants, from the B, N, and X endogenous viruses and from any combination thereof, and must therefore be recognized as a distinctive subclass of murine ecotropic viruses.

MATERIALS AND METHODS

Mice. C57BL/Ka mice of both sexes, 3–5 weeks of age, were obtained from our own colony. NIH/Swiss mice were kindly provided by Dr. Paul Arnstein, National Cancer Institute, California State Department of Health, Berkeley, Calif.

Viruses. RadLV was prepared as 20% extracts of RadLV-induced C57BL/Ka thymomas in phosphate-buffered saline, as previously described (1). Two such extracts prepared on 10-28-74 and 3-27-75 were used in these experiments. BL/Ka(6) virus was obtained from the supernatant of an established C57BL/Ka fibroblast line, BL-6 (RadLV), after more than 20 passages of that line *in vitro* following infection by RadLV. BL/Ka(B) clone 1 was derived from the supernatant of cultures of C57BL/Ka mouse embryo fibroblasts after infection by limiting dilutions of RadLV that had been treated with antiserum to Balb:virus-2. BL/Ka(N) was obtained from the supernatant of a culture of NIH/Swiss mouse embryo fibroblasts at the 20th passage after infection by a tissue extract of radiation-induced lymphoma (7). BL/Ka(X) was derived from mink lung cells that became productively infected after cocultivation with normal thymic cells from C57BL/Ka mice. AKR murine leukemia virus (MuLV) was isolated from an established line of AKR mouse embryo fibroblasts that spontaneously initiated virus replication. BL/6(B) and BL/6(N) viruses were isolated from SC-1 cells infected with endogenous C57BL/6 B- and N-tropic viruses, respectively. Balb/c(X) virus was obtained from cultures of mink lung cells infected with the xenotropic Balb:virus-2. The last three virus preparations were kindly provided by Dr. Janet Hartley (National Institutes of Health, Bethesda, Md.).

Cells. Primary and secondary cultures of mouse embryo fibroblasts (MEF) were prepared as described (10) from 13- to 15-day embryos of C57BL/Ka and NIH/Swiss mice. The established cell line of mink lung was kindly provided by Dr. A. J. Hackett, Naval Biomedical Research Laboratory, Oakland, Calif.

Infectivity Assays. The *in vitro* and *in vivo* immunofluorescence assays (11, 12) and the reverse XC plaque assay (13) have been described. For end-point titrations *in vitro*, the test cells were infected with virus and passaged for at least 15 generations, at which time an aliquot of the cells was analyzed by immunofluorescence. Another aliquot was seeded on XC cells, and plaques were counted 4 days later. The titration results represent the means of two or more separate experiments.

Neutralization Assays. All sera were heat-inactivated at 56° for 30 min. The sera included: (*a*) serum from normal 3- to 4-month-old C57BL/Ka mice; (*b*) pig anti-Balb:virus-2 (Ki-MSV) ("anti-xenotropic") serum, lot 4S-0422 from Huntington Labs, Brooklandville, Md.; (*c*) guinea pig anti-amphotropic 1504-A serum (kindly provided by Dr. R. V. Gilden, NCI Frederick Cancer Research Center, Frederick, Md.), which has a neutralization titer for the amphotropic 1504-A virus of 1:160 (14); (*d*) rabbit anti-AKR-MuLV gp71 ("anti-ecotropic") serum; and (*e*) rabbit anti-Rauscher-MuLV gp71, which has a neutralization titer for Rauscher virus of 1:1600. The last two sera were prepared using proteins purified from the respective viruses as described (15). Equal volumes of serial dilutions of antiserum were mixed with virus dilutions that would induce 20% immunofluorescence-positivity in the test cells for the neutralization assay *in vitro*, or as indicated in Table 3 for the neutralization assay *in vivo*. The mixtures of virus and serum were then held at room temperature for 1 hr and at 4° for another hour. Controls were treated in the same way in fetal calf serum (Microbiological Associates, Bethesda, Md.).

The serum-treated viruses were then injected intrathymically into animals or assayed in cell cultures *in vitro*. The results represent mean values of two or more separate experiments. Antibody titers are expressed as the reciprocal of the highest serum dilution that gave more than a 66% reduction in the percentage of immunofluorescence-positive cells, as compared to controls treated with fetal calf serum.

Abbreviations: RadLV, radiation leukemia virus; MuLV, murine leukemia virus; MEF, mouse embryo fibroblasts.

Table 1. *In vivo* and *in vitro* replication of virus isolates from strain C57BL/Ka mouse cells and tissues

Assay	Strain	Virus titers				
		RadLV 10-28-74	BL/Ka(6)	BL/Ka(B) clone 1	BL/Ka(N)	BL/Ka(X)
In vivo						
IF*	C57BL/Ka	3×10^9	1×10^4	0	0	0
IF*	NIH/Swiss	5×10^0	0	0	0	0
In vitro						
IF or reverse XC†	C57BL MEF	6×10^5	1×10^8	5×10^5	1×10^3	0
IF or reverse XC†	NIH MEF	4×10^0	1×10^2	$<1 \times 10^2$	6×10^6	0
IF†	Mink lung	1×10^3	1×10^3	0	0	1×10^6

* Immunofluorescence. End-point assay 3 weeks after intrathymic inoculation of the mice.
† End-point assay after 15 cell doublings of the test cells.

Radioimmune Precipitation Assays. Double-antibody radioimmune competition assays for viral antigens have been reported (16). The assay for AKR-MuLV gp71 used ^{125}I-labeled AKR-MuLV gp71 and its homologous antisera. The assays for p12 and p30 used ^{125}I-labeled AKR MuLV p12 and p30 and monospecific rabbit antisera prepared against these proteins. The assay for Balb:virus-2 gp71 used ^{125}I-labeled Balb:virus-2 gp71 and a goat antiserum prepared against C57L xenotropic virus purified by sucrose gradient centrifugation (kindly provided by Dr. R. V. Gilden, NCI Frederick Cancer Research Center, Frederick, Md.).

RESULTS

Replication of RadLV, BL/Ka(B), BL/Ka(N), and BL/Ka(X) Virus Isolates *In Vivo* and *In Vitro*. End-point titers (Table 1) indicate the relative ability of these isolates to replicate *in vivo* and *in vitro*. Confirming earlier observations (17), RadLV was found to be strongly thymotropic as well as B-tropic with respect to the Fv-1 host restriction system (18, 19). It grew to low titers on fibroblasts *in vitro*, but maintained its B-tropic nature. In addition, some xenotropic activity was detected on mink cells.

BL/Ka(6), also a B-tropic isolate, was, in contrast to RadLV, highly fibrotropic *in vitro* and replicated to low titers *in vivo*. Its xenotropic activity was similar to that of RadLV.

The BL/Ka(B) clone 1, BL/Ka(N), or BL/Ka(X) isolates displayed no evidence of replication in the thymus of C57BL/Ka or NIH/Swiss mice. Their growth patterns *in vitro* were typical of, respectively, B-, N-, and X-tropic viruses. When immunofluorescence-positive mink cells were tested in the reverse XC assay, plaque formation was not detected, confirming the observation by others (9) that xenotropic virus-infected cells do not form XC plaques.

Neutralization by Specific Antisera. From the infectivity experiments it was not clear whether RadLV and BL/Ka(6) were homogeneous virus isolates with multipotential infectivity or mixtures of viral subpopulations, each with its own infectivity pattern. The neutralization results observed *in vitro* (Table 2) indicate that the ecotropic activity of AKR-MuLV, BL/Ka(6), BL/Ka(B) clone 1, and BL/Ka(N) was readily neutralized by the anti-AKR-MuLV gp71 serum. Some crossreactivity with anti-Balb:virus-2 and anti-Rauscher gp71 could be observed; this has been previously reported by others (20, 21). The high neutralizing titer of the anti-Balb:virus-2 serum for BL/Ka(X) clearly established the xenotropic nature of this virus. This serum also neutralized the infectivity of RadLV and BL/Ka(6) on mink lung cells, confirming the presence of a xenotropic component in these preparations. Some neutralization of BL/Ka(X) was also observed with normal C57BL serum; this was reported by others (22, 23) to be due to a nonimmunoglobin factor (24, 25) that reacts specifically with xenotropic viruses.

It is evident that RadLV stands apart from other endogenous

Table 2. Neutralization by specific antisera *in vitro*

Virus	Test cells	Test sera*				
		Normal C57BL	Anti-AKR gp71	Anti-Balb:virus-2	Anti-amphotrope 1504-A	Anti-Rauscher gp71
AKR MuLV	NIH Swiss MEF	NT	512	64	NT	NT
RadLV(10-28-74)	C57BL/Ka MEF	<4	<4	<4	NT	NT
	Mink lung	NT	<4	>256	NT	NT
RadLV(3-27-75)	C57BL/Ka MEF	<4	<4	4	<4	16
BL/Ka (6)	C57BL/Ka MEF	<4	512	64	<4	32
	Mink lung	NT	<4	>256	NT	NT
BL/Ka(B)clone 1	C57BL MEF	NT	512	<32	NT	NT
BL/Ka(N)	NIH Swiss MEF	4	512	64	<4	32
BL/Ka(X)	Mink lung	32	<4	2,048	8	16
BL/Ka(6) in 20% thymus-spleen extract	C57BL/Ka MEF	<4	512	NT	NT	NT

* NT = not tested.

Table 3. Neutralization by specific antisera *in vivo* in C57BL/Ka mice

Virus	Dilutions	% IF*	Test sera†				
			Normal C57BL	Anti-AKR gp71	Anti-Balb:virus-2	Anti-amphotrope 1504-A	Anti-Rauscher gp71
RadLV 10-28-74	1:1 × 10⁵	15	0	8	64	NT	NT
	1:1 × 10⁶	4	0	8	64	NT	NT
RadLV 3-27-75	1:2 × 10⁵	7	0	4	32	<8	<8
BK/Ka(6)	1:10	15	NT	8	NT	<8	16
	1:20	11	NT	256	512	<8	32
	1:200	4	NT	512	1024	NT	NT

* Percentage of positive cells by immunofluorescence (IF) in the C57BL thymus 7 days after intrathymic injection of 1:8 mixtures of virus and fetal calf serum. Optimal plateau levels obtained with saturating concentrations of virus were 30% for RadLV 10-28-74 and 3-27-75 and 15% for BL/Ka(6).
† NT = not tested.

viruses, since no significant neutralization of its ecotropic infectivity was obtained with any of the specific antisera tested. That this is not due to protection of the virus by the high protein content of the tissue extract is indicated by the fact that the infectivity of RadLV on mink cells could be readily neutralized by the anti-Balb:virus-2 serum and that mixing BL/Ka(6) with 20% normal thymus-spleen extract did not prevent its neutralization by the anti-AKR gp71 serum.

In vivo studies (Table 3) confirmed that high dilutions of RadLV were not neutralized by the various sera tested, with the exception of the anti-Balb:virus-2 serum, which had slight neutralizing activity. At the 1:10 dilution, BL/Ka(6) virus, which shows low titers *in vivo*, could not be significantly neutralized by the antisera tested; higher dilutions were, however, neutralized by both the anti-AKR gp71 and the anti-Balb: virus-2 sera. The antiserum prepared against an amphotropic virus, 1504-A, of the wild mouse (14) did not neutralize any of the virus preparations tested *in vivo* or *in vitro*.

Radioimmune Competition Assay. The serological characteristics of RadLV, BL/Ka(6), BL/Ka(N), and BL/Ka(X) viruses were compared to those of other virus isolates by radioimmune competition assays. Viruses purified by sucrose gradient centrifugation or extracts of cells replicating the appropriate virus were used. The results of assays using radioiodinated AKR-MuLV gp71 and antiserum against this glycoprotein are illustrated in Fig. 1A. Whereas the BL/Ka(6), BL/Ka(N), BL/6(N), and BL/6(B) isolates competed completely and equivalently against AKR-MuLV gp71, no competition was observed with the two RadLV preparations, or with the BL/Ka(X) and Balb/c xenotropic viruses. This demonstrates that the glycoprotein of RadLV is serologically distinct from that of the endogenous ecotropic, fibrotropic viruses. Fig. 1B illustrates the results obtained in competition assays using radioiodinated AKR-MuLV p30. Complete and equivalent competition was observed for all of the virus preparations. In Fig. 1C, the results obtained with a competition assay for AKR-MuLV p12 are presented. Only BL/Ka(N) and the BL/6(N) viruses competed completely and equivalently against AKR-MuLV p12. This was expected since this p12 is type-specific for (N)-ecotropic viruses (J. N. Ihle, unpublished observation). Thus, the results of radioimmune competition with the AKR-MuLV gp71, p30, and p12 standards demonstrate that BL/Ka(6) and BL/Ka(N) compete as standard endogenous B- and N-ecotropic, fibrotropic viruses, that the BL/Ka(X) preparation is homogeneously xenotropic, since it does not contain detectable levels of ecotropic gp71 antigen, and that RadLV seems to be clearly different from the endogenous fibrotropic viruses, as demonstrated by its lack of competition with AKR-MuLV gp71 glycoprotein.

In competition assays using radioiodinated Balb:virus-2 gp71 and an antiserum against C57L xenotropic virus, Balb:virus-2 xenotropic virus competed completely, whereas AKR virus, BL/Ka(6) virus, and cell extracts of BL/Ka(N) virus-producing cells competed poorly against Balb:virus-2 gp71 (Fig. 2). Cell homogenates of BL/Ka(X)-producing cell lines and RadLV both competed against Balb:virus-2. Similar results were obtained with an extract of cells productively infected by the BL/Ka(6) virus (data not shown). These results are consistent with the evidence that the RadLV and BL/Ka(6) preparations contain xenotropic virus, as demonstrated by their infectivity for mink cells *in vitro* and by the inhibition of this infectivity by neutralizing antisera against xenotropic virus.

DISCUSSION

Several viruses present in C57BL/Ka mice have been described in these studies. The isolates BL/Ka(B) clone 1, BL/Ka(N), and BL/Ka(X) appear homogeneous on the basis of their infectivity on fibroblastic cells *in vitro*, their susceptibility to specific neutralizing antisera, and their serological characteristics in radioimmune competition assays. The ability of antisera against AKR-MuLV gp71 to specifically neutralize BL/Ka(B) clone 1 and BL/Ka(N), and the detection of this glycoprotein by competition assays, demonstrate that these viruses are ecotropic viruses. Moreover, the detection of AKR-MuLV p12 in BL/Ka(N) but not in BL/Ka(6) isolates provides a serological difference associated with the N- and B-tropic infectivity patterns of the virus preparations *in vitro*. Similarly, the BL/Ka(X) virus appears to be serologically equivalent to other xenotropic isolates, based on infectivity, neutralization, and detection of the Balb:virus-2 type of glycoprotein in competition assays. Perhaps the most interesting characteristic of these apparently "classical" endogenous, fibrotropic viruses, however, is that none of them initiates productive infection or neoplastic transformation *in vitro* in C57BL/Ka mouse thymocytes.

Two of the preparations studied, RadLV and BL/Ka(6), were clearly mixtures of viruses. Both contained a xenotropic virus capable of replicating on mink lung cells and inhibited specifically by antisera to xenotropic virus. Moreover, RadLV and BL/Ka(6)-infected cells (data not shown) were found by radioimmune competition assay to contain a glycoprotein serologically equivalent to that of the Balb:virus-2 gp71. The major

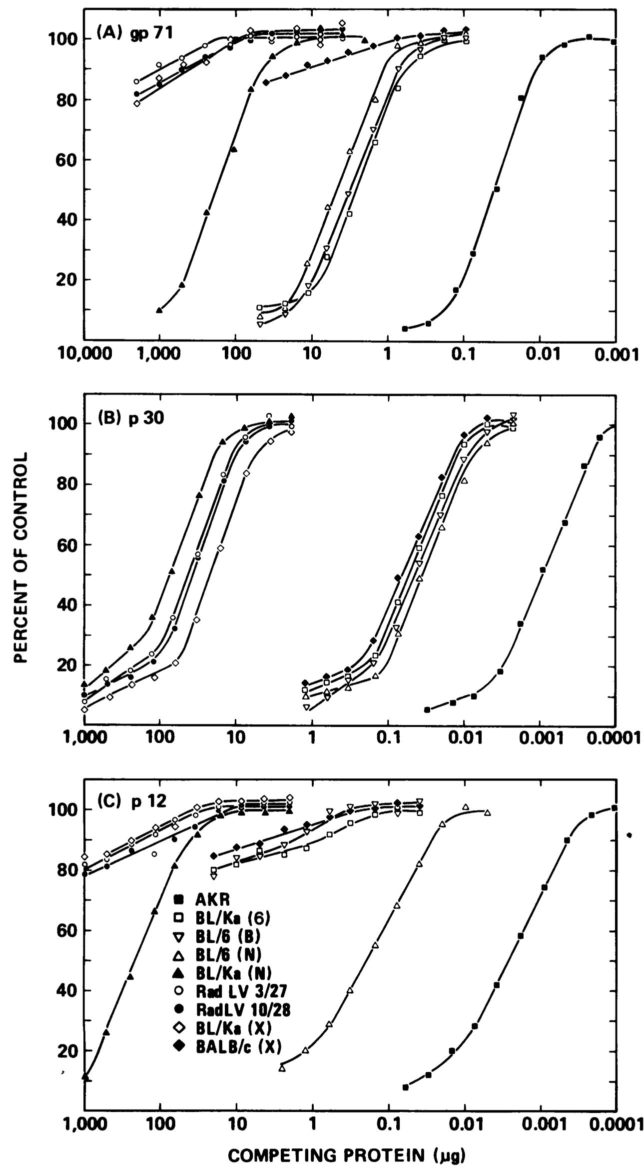

FIG. 1. Radioimmune competition assays for AKR-MuLV virion proteins. Competition assays using: (A) ^{125}I-labeled AKR-MuLV gp71 and monospecific rabbit antiserum against this component; (B) ^{125}I-labeled AKR-MuLV p30 and a monospecific rabbit antiserum against p30; (C) ^{125}I-labeled AKR-MuLV p12 and a monospecific rabbit antiserum against AKR-MuLV p12. Competing samples included cell extracts of tissue culture cells producing BL/Ka(X) and BL/Ka(N) viruses; thymus extracts of RadLV, 3/27 and 10/28; viruses purified by sucrose gradient centrifugation, including BL/Ka(6), BL/6(B), BL/6(N), and Balb/c(X), and purified AKR-MuLV gp71, p30, and p12.

component of the preparation of BL/Ka(6) virus was a virus serologically and biologically equivalent to a B-ecotropic virus. This virus replicated preferentially on C57BL/Ka fibroblasts, was completely inhibited by antisera to AKR-MuLV gp71, and contained a glycoprotein serologically indistinguishable from AKR-MuLV gp71 by competition assays. In contrast, RadLV contains, in addition to its minor xenotropic component, a virus population serologically distinct from all the other viruses tested and uniquely capable of infecting and replicating to high titer in C57BL/Ka mouse thymocytes. This virus could be detected only at low titer on C57BL/Ka fibroblasts *in vitro*. Neither activity could be abolished by neutralizing antisera to ecotropic,

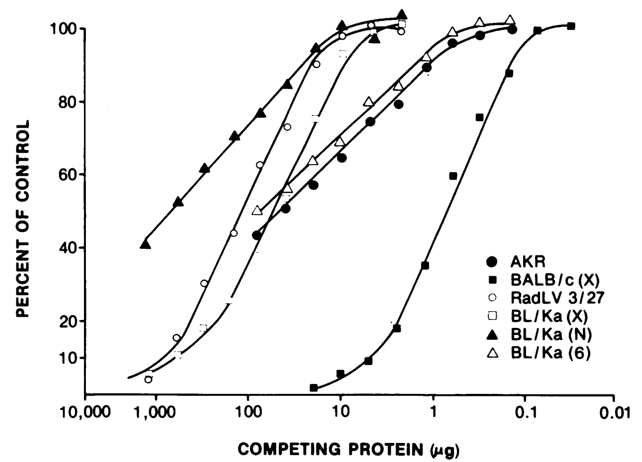

FIG. 2. Radioimmune competition assays for Balb:virus-2 gp71. Competition assays used ^{125}I-labeled Balb:virus-2 gp71 and goat antiserum against C57L xenotropic virus purified by sucrose gradient centrifugation. Competing samples included thymus extracts of RadLV (3/27); cell extracts of tissue culture cells replicating BL/Ka(N) and BL/Ka(X); and the purified viruses BL/Ka(6), AKR-MuLV, and Balb/c(X).

xenotropic, or amphotropic viruses. No B-ecotropic-fibrotropic component could be detected in the RadLV preparation.

The *in vivo* activity of BL/Ka(6) at low dilutions was similar to that of RadLV in that it was not inhibited by neutralizing antisera against ecotropic or xenotropic viruses. In contrast, however, this activity was progressively inhibited by both antisera at higher dilutions. Although the basis for this difference is not known, BL/Ka(6) may also contain low levels of the thymotropic component of RadLV detectable only at high virus concentrations. At higher dilutions, the co-existence of B-ecotropic and xenotropic viruses in BL/Ka(6) may account for residual thymotropism, both viruses being required for infectivity, as indicated by the neutralization studies. Clearly, however, this is not the case with RadLV, which contains a fully competent thymotropic and leukemogenic population of virions.

When RadLV was first adapted to propagation *in vitro* on C57BL MEF cells (26), it was noted that the progeny virus, after several passages, had lost all its leukemogenicity. This was initially interpreted as the result of "modulation" or selection in the intracellular environment of the fibroblast (26). Haas and Hilgers (27) found that RadLV propagated on lymphoma cells *in vitro* lacked fibrotropic activity but retained its thymotropic activity. They suggested the alternative hypothesis that RadLV may be composed of a heterologous mixture of two different B-ecotropic viruses, one thymotropic and the other fibrotropic. Of particular importance in the present studies is the demonstration that RadLV contains no detectable B-ecotropic, fibrotropic viral component, despite the fact that such a virus can be isolated from murine fibroblastic cultures infected with RadLV. This is evident from the fact that antiserum against AKR MuLV gp71 does not inhibit RadLV "infection" of cultures of C57BL/Ka MEF, although it completely inhibits the ability of the emergent virus [BL/Ka(6)] to replicate on cultures of C57BL/Ka MEF. These results can best be interpreted as indicating that the thymotropic RadLV induces the expression of the endogenous B-ecotropic, fibrotropic virus *in vitro*. Whether this "induction" involves complete *de novo* induction of the virus, as has been reported by others (28), or is due to a complementation-rescue mechanism is not known. Clearly, however, if this interpretation is correct, the *in vitro* assay of

RadLV on C57BL/Ka MEF cultures is an assay of a biological effect rather than replication since the resulting virus preparations have lost both the serological characteristics associated with RadLV and the ability to induce thymic lymphomas.

The apparent serological uniqueness of the thymotropic, leukemogenic RadLV is particularly relevant to recent studies of virus expression in primary, radiation-induced thymic lymphomas in C57BL/6 mice (29). In particular, these studies demonstrated that there was no serological correlation between a humoral immune response against endogenous ecotropic fibrotropic viruses and leukemia. Moreover, using competition assays and RNA·DNA hybridization, there was no evidence of overt ecotropic or xenotropic virus expression in the majority of thymomas. These studies must now be re-examined after the serological characteristics of RadLV are determined and the assays for the specific detection of this virus are developed. The relationship of RadLV with the AKR Gross virus in terms of its serological properties (30, 31) will also require reconsideration in the light of the present data.

Finally, it is clear that a differentiation-specific restriction system governing the replication of the murine ecotropic C-type viruses operates in addition to the existing Fv-1 and SRV gene restriction systems. The thymotropic-leukemogenic viruses replicate selectively in mouse thymic lymphocytes *in vivo*, whereas viruses of the other subclass replicate preferentially in mouse fibroblasts *in vitro*. However, both subclasses are subject to control by the Fv-1 (18, 19) and SRV (17) genes. These findings focus attention on the desirability of propagating candidate leukemia-lymphoma viruses in lymphoid rather than fibroblastic cells to preserve their leukemogenicity. Moreover, it no longer seems appropriate to use the term "murine leukemia viruses" for C-type murine viruses that have not been shown to be leukemogenic.

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