RADIATION LEUKEMIA IN C57BL/6 MICE

II. Lack of Ecotropic Virus Expression in the Majority of Lymphomas*

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In the accompanying paper we examined the relationship between the immune response against endogenous murine leukemia virus(es) (MuLV)[1] and the development of lymphoma in irradiated C57BL/6 mice (1). The results demonstrated that irradiation accelerated the appearance of antibody against MuLV, supporting the hypothesis that irradiation activates the expression of endogenous ecotropic virus. However, no correlation between the development and persistence of this humoral immune response against MuLV and the subsequent development of lymphoma was demonstrated. The lack of correlation with immunity could have been due to differing degrees of viremia associated with the lymphoma; therefore, in the present experiments we examined the levels of virus expression, using a variety of assays. The results demonstrate that only occasionally can overt ecotropic virus antigen or RNA expression be detected in radiation-induced thymomas.

Materials and Methods

Mice. Male C57BL/6 mice were used in these experiments. All mice were specific pathogen free and were obtained from the central animal facility of the Frederick Cancer Research Center, Frederick, Md.

Radiation. Mice were irradiated at 1 mo of age with 175 R of whole-body irradiation four times at weekly intervals. The source was a Phillips MG 301 X-ray therapy unit (Phillips Electronic Instruments, Mount Vernon, N. Y.) operated at 10 mA and 300 kV with a 0.2 mm Cu filter.

Viruses. Friend MuLV was obtained from the Eveline cell line, which was derived from the STU mouse strain (2). AKR MuLV was isolated from an established line of AKR mouse embryo cells that spontaneously initiated virus synthesis. Xenotropic viruses from NZB and BALB/c mice were obtained from cultures of mink lung cells infected by the appropriate viruses. These viruses were kindly provided by Dr. J. Hartley, NIH, Bethesda, Md., who also provided lines of Sc-1 cells infected with endogenous C57BL/6 N- and B-tropic viruses for production of these viruses. Radiation leukemia virus (RADLV) was isolated from C57BL/Ka fibroblast cultures infected with in vivo derived RADLV. These cultures were at passage 11 after infection when used, and were kindly provided by Dr. A. DeCleve, Stanford University, Stanford, Calif. All viruses were purified by velocity and equilibrium density centrifugation, as previously described (3, 4).

Viral Antigens. AKR MuLV gp71 and AKR MuLV p30 were purified as previously described (5). AKR MuLV p12 was purified in a similar manner to p30, except it was eluted from DEAE-Sephadex with 0.5 M NaCl after the elution of p30. Rechromatography on DEAE-Sephadex yielded

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[1] *Abbreviations used in this paper:* MuLV, murine leukemia virus(es); NP-40, Nonidet P-40; RADLV, radiation leukemia virus; SDS, sodium dodecyl sulfate.

a homogeneous antigen preparation as determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

Antisera. Antisera to virus-specific proteins were prepared in rabbits as previously described (4). Antiserum (IgG fraction) to purified reverse transcriptase from Rauscher MuLV and a preimmune serum were kindly provided by Dr. Robert Gallo, NIH. Antiserum against Friend MuLV gp71 was kindly provided by Dr. Dani Bolognesi, Duke University, Durham, N. C.

Radioimmune Competition Assays. Viral antigens were iodinated with ^{125}I with chloramine T (6). Specific activities were generally 1–5 × 10^4 cpm/ng. Tissue extracts were prepared by homogenizing either washed single cell suspensions or intact tissue. Single cell suspensions were obtained by pushing organs through a wire mesh screen while constantly washing with Eagle's minimum essential medium, filtering the suspension through gauze, and then pelleting the cells by centrifugation. Red blood cells were subsequently removed by resuspending the cells in 0.85% ammonium chloride for 10 min at 20°C, and intact cells were removed by centrifugation. Comparable results were obtained with either cell suspensions or tissue homogenates, although extracts of single cell suspensions interfered less in the assays at high protein concentrations. In either case, an equal volume of 1% Triton, 1 M KCl, and 0.1 M sodium phosphate buffer at pH 7.0 was added, and the cells or tissues were homogenized with 10 strokes in a ground glass homogenizer. One volume of H_2O was added and the tissue rehomogenized. The extract was then centrifuged at 15,000 rpm for 20 min, and the supernate aliquoted and frozen at −20°C for assays.

Competition radioimmune precipitation assays were comparable to those previously reported (5). Tissue extracts were diluted twofold in 0.2 ml of 0.01% Triton, 0.1 M NaCl, and 0.05 M Tris-HCl, pH 7.5. Appropriate concentrations of monospecific rabbit antisera to either AKR MuLV gp71, p30, or p12 and approximately 0.01 ml of normal rabbit gamma globulins were added. The mixtures were incubated 1 h at 37°C, iodinated antigen was added, and the mixtures incubated 3 h at 37°C and overnight at 4°C. Goat antisera to rabbit gamma globulins were then added at the appropriate concentration to reach the equivalence point for precipitation of the added carrier rabbit gamma globulins and incubated 2 h at 37°C and 3 h at 4°C. Immune precipitates were then collected by centrifugation, washed once with the above buffer, and counted in a gamma counter. The results are presented as the percent of labeled antigen precipitated relative to that precipitated in the absence of competing antigen.

Serum Cytotoxicity. Serum-mediated complement(C)-dependent cytotoxicity was assayed with a chromium release assay. Briefly, 10^7 target cells were labeled with 100 μCi ^{51}Cr (Na_2CrO_4) in 1 ml Ham's F-12 medium at 37°C for 1 h. The cells were washed three times with the same medium and finally resuspended at a concentration of 1 × 10^6 cells/ml. 100 μl containing 10^5 cells were added to 0.4 ml of medium containing the appropriate test serum dilution in 12 × 75 mm plastic tubes. The mixture was incubated at 37°C for 1 h after which guinea pig C was added at a 1:20 final concentration. The cultures were further incubated for 1 h at 37°C, the tubes were centrifuged at 1,000 rpm for 10 min, and the supernate was decanted into a scintillation vial containing 7 ml of Aquasol-2 (Wayne Chemical Products Co., Detroit, Mich.) and counted.

Percent lysis was calculated as:

$$\frac{\text{cpm with antibody} - \text{cpm with Ham's F-12 only}}{\text{total releasable cpm target} - \text{cpm with Ham's F-12 only}} \times 100.$$

Preparation of Viral RNA. Viral 70S RNA was isolated from purified virus by phenol extraction and sucrose gradient centrifugation, as previously described (7).

Preparation of Tissue RNA. Cells or tissues were suspended in five to seven volumes of 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, and 50 mM NaCl and homogenized with 10 strokes in a ground glass homogenizer at 4°C. The suspension was made 0.5% Nonidet P-40 (NP-40)(Shell Chemical Co., New York), mixed, and centrifuged at 1,000 *g* for 10 min. The supernate was adjusted to contain 0.5% SDS and extracted with an equal volume of cold water-saturated phenol. The aqueous layer was removed and the phenol-protein interphase was extracted twice with 0.1 M Tris-HCl, pH 9.0. The pooled aqueous phases were then extracted twice with cold phenol and the RNA was precipitated with 2.5 volumes of ethanol. RNA was collected by centrifugation, washed with alcohol, dried, and dissolved in hybridization buffer. The RNA preparations contained intact ribosomal RNA and the contamination with DNA was less than 5%.

Preparation of Virus-Specific [^3H]cDNA. [^3H]cDNA was synthesized with the endogenous

reverse transcriptase reaction. Virus was harvested at 3-4 h intervals, banded in sucrose, and collected by centrifugation (4, 7). The virus pellet was suspended in 2.0 ml of 0.05 M Tris-HCl, pH 8.0, 0.05 M KCl, 0.05% NP-40, 5 mM dithiothreitol, 2.0 mM $MnCl_2$, 250 μg/ml bovine serum albumin, 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.01 mM [^{3}H]dTTP (27 Ci/mmol), and 100 μg/ml actinomycin D. After incubation at 37°C for 6 h the mixture was made 0.5% SDS and deproteinized by three phenol extractions and one extraction with chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated with 2.5 volumes of ethanol, collected by centrifugation (100,000 *g* for 1 h), dissolved in 0.5 ml of 0.4 M KOH, and incubated at 23°C for 20 h to hydrolyze RNA. Sheared, single-stranded calf thymus DNA (100 μg) was added as a carrier and the solution was neutralized with HCl. The sample was applied to a 1.5 × 55 cm Sephadex G-50 column, and equilibrated with 0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, and 1 mM EDTA. The [^{3}H]cDNA eluting in the void volume was pooled, precipitated with ethanol, collected by centrifugation, and dissolved in H_2O. The [^{3}H]cDNA product had a sp act of 1-2 × 10^7 cpm/μg and was totally digested by nuclease S_1. AKR viral [^{3}H]cDNA prepared by this procedure protects 90% of AKR [^{125}I]RNA from S_1 and RNaseA digestion.

DNA-RNA Hybridization. Cellular or viral RNA and [^{3}H]cDNA (2,000 cpm) were heated at 95°C for 5 min, and incubated for 48 h at 62°C in a 0.055 ml reaction mixture containing 0.01 M Tris-HCl, pH 7.3, 0.45 M NaCl, 0.1 mM EDTA, and 0.5% SDS. The DNA-RNA hybrids that formed were analyzed with S_1 nuclease, which degrades single-stranded DNA and RNA, but not DNA-RNA hybrids. The hybridization mixture was adjusted to contain in 0.11 ml, 0.1 M sodium acetate buffer, pH 4.6, 0.3 M NaCl, 0.5 mM Zn SO_4, 5% glycerol, and enough S_1 to hydrolyze greater than 99% of the single-stranded cDNA. Each mixture was incubated at 37°C for 1.5 h; spotted on a Whatman 3-mm paper disk; washed with 5% TCA, ethanol, and ether; and the radioactivity measured. During the hybridization reaction without RNA the [^{3}H]cDNA probe remained greater than 99% single stranded. The percent hybridization is defined as the counts per minute resistant to S_1 nuclease divided by the total acid precipitable counts per minute.

Results

To examine the levels of ecotropic virus antigen expression in radiation-induced lymphomas, we used radioimmune competition assays for gp71, p30, and p12. The serological specificities of these assays are shown in Fig. 1. Panel A illustrates the results obtained with a competition assay using labeled AKR MuLV gp71 and a monospecific antiserum against this glycoprotein. In this assay, in vitro passaged RADLV and an N-tropic C57BL/6 virus competed completely and equivalently to purified AKR MuLV gp71. In contrast, Friend MuLV, an X-tropic virus from NZB mice (NZB-X) and an X-tropic virus from BALB/c mice (BALB/c-X) did not compete significantly. Thus, this assay is specific for the expression of endogenous ecotropic virus glycoprotein. The characteristics of an assay for p30 using labeled AKR MuLV p30 and an antiserum against Rauscher MuLV p30 are shown in panel B. In this assay all the viruses competed equivalently, thus providing an assay for the expression of both ecotropic and xenotropic viruses. The results obtained with an assay using labeled AKR MuLV p12 and a monospecific antiserum to AKR MuLV p12 are shown in panel C. In this assay the N-tropic virus isolated from C57BL/6 mice competed completely and identically to the standard curve obtained with purified AKR MuLV p12. In contrast, RADLV, BALB/c-X and NZB-X competed only slightly and Friend MuLV did not compete. Thus, this assay can distinguish between the N- and B-tropic viruses of C57BL/6 mice. These results are comparable to those previously described for p12 which demonstrated the highly type-specific serological properties of this virion polypeptide (8).

The results obtained with C57BL/6 thymomas are shown in Fig. 2. Panel A

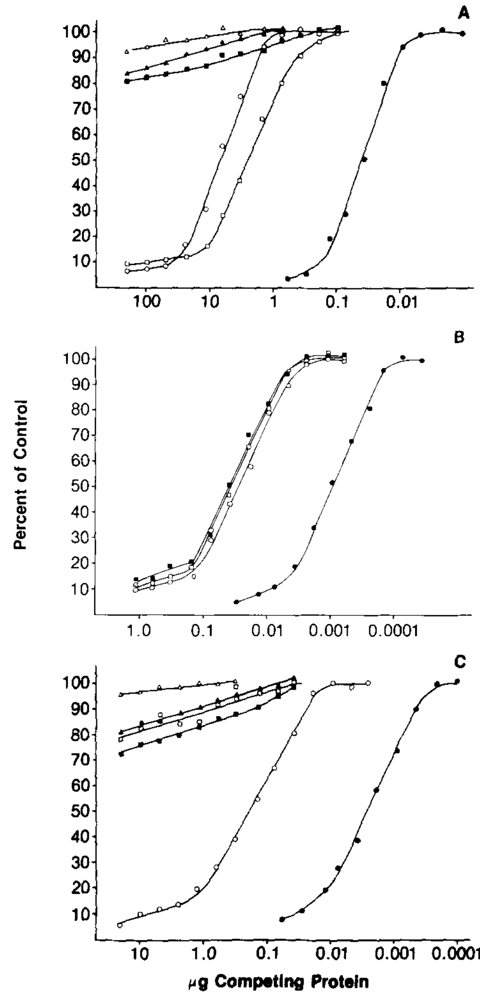

FIG. 1. Radioimmune competition assays with purified viruses. Radioimmune competition assays for AKR MuLV gp71 (A), p30 (B), and p12 (C) were performed as described in Materials and Methods using a C57BL/6 N-tropic virus isolated from Sc-1 cells, (○ – ○); in vitro derived RADLV, (□ – □); a xenotropic virus from BALB/c mice replicating in mink lung cells, (■ – ■); a xenotropic virus from NZB mice replicating in mink lung cells, (▲ – ▲); or Friend MuLV (△ – △). Standard competition curves (● – ●) were obtained with purified AKR MuLV gp71, p30, or p12 in A, B, and C, respectively. In B, competition curves for Friend MuLV, NZB (X) were identical to those obtained with the other viruses and are not plotted.

illustrates typical results obtained with the competition assay for AKR MuLV gp71. In general, most of the 40 thymoma extracts examined gave either no competition or only weak competition with slopes distinctly less pronounced than those for AKR MuLV gp71. However, occasionally thymomas were found that yielded extracts having significant levels of gp71, as illustrated by the results of one such extract in Fig. 2. Moreover, the competition curves obtained with these extracts were identical in extent and slope to those obtained with

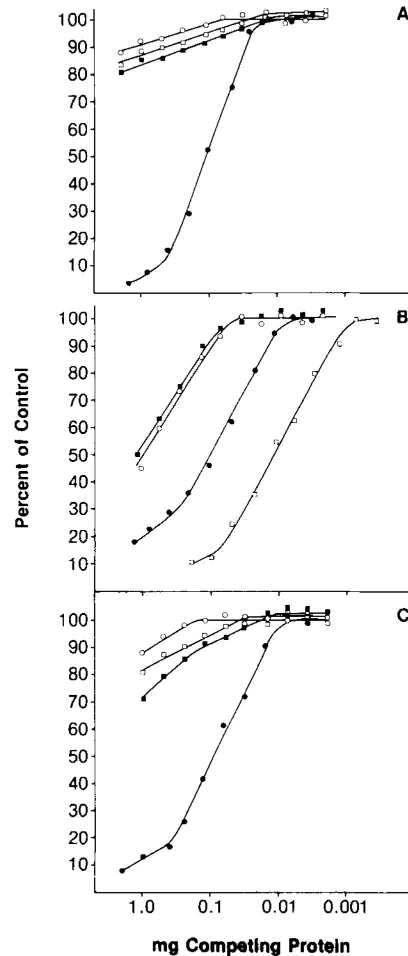

FIG. 2. Radioimmune assays of thymus extracts from leukemia C57BL/6 mice. Radioimmune competition assays for AKR MuLV gp71 (A), p30 (B), and p12 (C) were performed as described in Materials and Methods. The results shown were obtained with extracts from three leukemic C57BL/6 mice (●–●, □–□, ■–■) and from a normal unirradiated, age-matched control (○–○).

AKR MuLV gp71. In general, thymus extracts from control, nonirradiated mice failed to compete.

Typical results obtained with thymoma extracts in competition assays for p30 are shown in Fig. 2B. In contrast to the results with gp71, all the 40 thymomas examined had low but detectable levels of competing antigen. Occasionally, extracts had relatively high levels of competing antigen and, in general, the slope and extent of competition were comparable to that of standard p30. Similar to the results obtained with the thymomas, age-matched control thymus extracts also consistently yielded low levels of competing antigen. These results are in accord with previous reports of a low level of p30 expression in a variety of inbred strains of mice (9, 10) and could suggest the

constitutive expression of endogenous xenotropic virus as also indicated by the hybridization data (below).

Typical results of competition assays for p12 are shown in Fig. 2 C. Comparable to the results with AKR MuLV gp71, the majority of thymomas yielded extracts that competed only weakly and had slopes distinctly less pronounced than the standard p12. However, one thymoma extract was obtained which competed completely and equivalently to the standard p12. Interestingly, this thymoma extract also had detectable gp71, although two thymomas that had detectable gp71 had no detectable p12. These results suggest the presence of both N- and B-tropic viruses in these thymomas. Thymic extracts from control, nonirradiated mice generally did not compete in this assay.

Typical results of these and additional assays are summarized in Table I. In general, most lymphomas were characterized by the absence of gp71, low levels of p30, and the absence of p12. Furthermore, these results were comparable to those obtained with age-matched control, nonirradiated mice; and, in general, the two groups were indistinguishable. In addition, a small group of thymomas were characterized by significantly elevated levels of p30 and readily detectable levels of p12 and/or gp71. Out of the 40 thymomas examined in this experimental group, 3 had detectable ecotropic gp71, 1 had detectable p12, and 10 had p30 levels higher than 10 ng/mg protein. These results demonstrate that overt ecotropic viral antigen expression is rare in radiation-induced lymphomas of C57BL/6 mice.

To further examine the expression of MuLV-related antigens in C57BL/6 thymomas, we then questioned whether rabbit antisera to AKR MuLV p12 or gp71 and antisera to Friend MuLV gp71 were cytotoxic against thymoma cells. The results are shown in Table II. Antiserum to AKR MuLV p12 is cytotoxic against cells replicating AKR MuLV as demonstrated here by the lysis of thymocytes from leukemic AKR mice. However, this serum was not cytotoxic against thymocytes from normal BALB/c, 129/J, or NIH Swiss mice. This serum was also not cytotoxic against any of the C57BL/6 leukemias tested. Antiserum against AKR MuLV gp71 is highly type specific and neutralizes only Gross or AKR viruses, whereas the antiserum against Friend MuLV gp71 is broadly reactive and can neutralize Friend-Moloney-Rauscher-, xenotropic-, and Gross-type viruses (Ihle, Fischinger, and Bolognesi, unpublished observations). Although these sera were strongly cytotoxic against AKR leukemic thymocytes, they were only weakly and inconsistently cytotoxic against normal NIH Swiss, BALB/c, or 129/J thymocytes. Similarly, only weak and inconsistent cytotoxicity was detectable against leukemic thymocytes from C57BL/6 mice. These results are in agreement with the competition assays and further demonstrate the infrequent expression of ecotropic viral antigens on C57BL/6 thymomas.

To further analyze the extent of virus expression in radiation-induced thymomas we examined a few tissue extracts for the presence of reverse transcriptase after chromatography on poly G-Sepharose. This technique has been shown to separate cellular reverse transcriptase from DNA polymerase α and β and provides a rapid means of purifying the enzyme from cellular extracts

TABLE I
*Ecotropic Virus Antigen Expression in Control and Tumor-Bearing C57BL/6 Mice**

Mouse no.	Tumor	ng/mg Protein		
		gp71	p30	p12
1	−	<1.0	2.2	<0.2
2	−	<1.0	3.2	<0.2
3	−	<1.0	6.4	<0.2
4	−	<1.0	5.8	<0.2
5	−	<1.0	9.1	<0.2
6	+	100	12.5	8.7
7	+	300	29.0	<0.2
8	+	689	20.0	<0.2
9	+	<1.0	5.5	<0.2
10	+	<1.0	10.0	<0.2
11	+	<1.0	30.0	<0.2
12	+	<1.0	6.7	<0.2
13	+	<1.0	3.6	<0.2
14	+	<1.0	1.0	<0.2
15	+	<1.0	3.3	<0.2

* C57BL/6 mice were irradiated at 1 mo of age with 175 R four times at weekly intervals. Control, nonirradiated mice were 7 mo of age. Antigen levels were determined by competition radioimmune precipitation assays as described in Materials and Methods, using either thymus or thymoma extracts. In general, comparable values were obtained with spleen extracts. Values of <1.0 and <0.2 for gp71 and p12 indicate no detectable competition and represent the approximate limits of the assays.

(11). Fig. 3 illustrates the elution profile from poly G-Sepharose of DNA polymerase activity from extracts of a spontaneous AKR thymoma and two C57BL/6 radiation-induced thymomas. All tissue extracts had a major peak of activity eluting at 0.25 M NaCl with preferential activity with poly (rA)·(dT) as a template. This activity corresponds to DNA polymerase β. With the AKR thymoma, a second major peak of activity eluted at 0.4 M NaCl corresponding to the elution position of reverse transcriptase. In contrast, only a minor peak of activity was eluted at 0.4 M NaCl from a comparable extract from a radiation-induced thymoma (Fig. 3 B). This peak of activity was comparable to that found in thymus extracts from control nonirradiated mice. Since DNA polymerase elutes from this column at a position comparable to reverse transcriptase, we examined the effect of specific antisera to reverse transcriptase on this enzyme activity. As seen in Table III, this antiserum significantly inhibited the reaction of AKR MuLV reverse transcriptase and the activity eluting at 0.4 M NaCl from AKR thymomas, but did not inhibit any of the activity from the C57BL/6 thymoma. The results of a second thymoma extract are also shown in Fig. 3 C and in Table III. This particular thymoma was examined because competition assays for gp71 suggested the presence of ecotropic virus. The results demonstrated that reverse transcriptase was detectable in this thymoma by its elution from poly G-Sepharose, and this activity was partially inhibited by the antiserum to reverse transcriptase. These results, therefore, are similar to the competition assays and demonstrate that overt virus expression is not consistently detectable in C57BL/6 thymomas.

TABLE II
Cytotoxicity of Xenogenic Antisera to Virus-Specific Antigens Against AKR and C57BL/6 Thymomas

	Target cell	Antiserum against*					
		AKR MuLV p12		AKR MuLV gp71		Friend MuLV gp71	
		1:40	1:80	1:40	1:80	1:40	1:80
Exp. 1	Normal BALB/c thymocytes	0	0	8	9	6	0
	AKR leukemic thymocytes	79	80	96	100	99	89
	C57BL/6 leukemic thymocytes	0	0	0	0	29	4
Exp. 2	AKR leukemic thymocytes	42	ND‡	54	ND	59	ND
	C57BL/6 leukemic thymocytes	0	ND	3	ND	5	ND
	Normal 129/J thymocytes	8	ND	29	ND	2	ND
Exp. 3	AKR leukemic thymocytes	101	69	92	74	98	93
	Normal NIH thymocytes	0	0	30	14	4	3
	C57BL/6 leukemic thymocytes	1	0	9	0	7	3
	C57BL/6 leukemic thymocytes	0	0	8	0	0	2
	C57BL/6 leukemic thymocytes	6	0	10	0	3	2
	C57BL/6 leukemic thymocytes	0	0	34	13	3	1

* C-dependent cytotoxicity assays were performed as described in Materials and Methods. C57BL/6 leukemic thymocytes were from mice irradiated at 1 mo of age with 175 R four times at weekly intervals. AKR leukemic thymocytes were obtained from AKR mice approximately 7-mo old that had developed spontaneous thymic lymphomas. Thymocytes were obtained from untreated, control BALB/c, 129/J, and NIH Swiss mice of approximately 3 mo of age. Cytotoxicity assays using thymocytes from control, nonirradiated, age-matched control C57BL/6 mice gave values comparable to those obtained with leukemia thymocytes.

‡ ND, not determined.

To further examine the extent of C-type virus expression in radiation-induced lymphomas, we examined the levels of viral RNA detectable by cDNA hybridization. The results are illustrated in Fig. 4 and tabulated in Table IV. Fig. 4 A illustrates the results obtained with AKR MuLV cDNA and 70S RNA. This probe hybridized 90% with AKR MuLV 70S RNA and approximately 50% with AT-124 xenotropic 70S RNA. These results are in agreement with previous studies of the relationship of ecotropic and xenotropic viruses by hybridization (12). Fig. 4 B illustrates the results obtained with RNA from normal and leukemic C57BL/6 thymomas, as well as the results obtained with RNA from an AKR thymoma. In general, the levels of RNA sequences hybridizing with AKR MuLV cDNA in C57BL/6 thymomas were about 100-fold below the concentrations detectable in AKR thymomas. However, occasionally thymomas were examined that had RNA concentrations approximating those of AKR thymomas. As expected, the thymoma illustrated in Fig. 4 B, that had high levels of RNA, also had readily detectable AKR MuLV gp71 and AKR MuLV p12 by competition assays. Although most thymomas had low levels of detectable RNA, these levels were three- to five-fold higher than those found in age-matched control thymus extracts with the exception of one thymoma in which the concentration was approximately the same.

To further examine the relationship of the sequences detectable by hybridi-

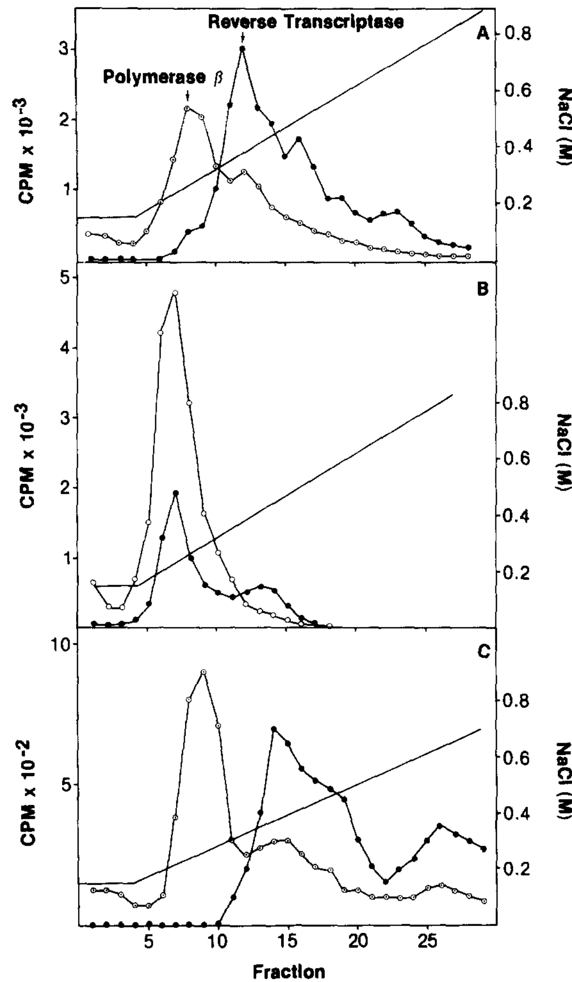

FIG. 3. Chromatography of leukemic thymus extracts on poly G-Sepharose. Thymus extracts were prepared from a leukemic AKR (A) and from radiation-induced leukemic C57BL/6 mice (B and C) by high salt-detergent extraction as previously described (10). After dilution with H_2O the extract was applied to a 0.6 × 7.0 cm poly G-Sepharose column and the column was developed with a linear NaCl gradient (0.15–1.0 M) as described (10). Aliquots of each fraction were assayed for DNA polymerase activity with poly (rA)·poly (dT), (○—○); or poly (rA)·$(dT)_{10}$ (●—●) as templates.

zation with AKR MuLV, the melting temperatures of the hybrids were examined (Fig. 5, Table IV). Hybrids between AKR MuLV cDNA and 70S RNA from AKR MuLV or AT-124 xenotropic virus melted at 80°C and 73°C, respectively. This difference is comparable to those previously reported (12) for RNA-DNA hybrids between ecotropic and xenotropic viruses. Moreover, RNA-DNA hybrids with RNA from AKR thymomas, AKR fibroblasts, or C57BL/6 fibroblasts replicating RADLV melted at 77–80°C. In contrast, RNA-DNA hybrids with RNA from the majority of radiation-induced C57BL/6 thymomas having low levels of hybridizing sequences melted at 72–75°C. Of the thymomas that

TABLE III

*Inhibition of DNA Polymerase Activity from Poly G-Sepharose with Antiserum to Rauscher MuLV Reverse Transcriptase**

Enzyme fraction	EU‡ assayed	Inhibition
		%
AKR MuLV reverse transcriptase	6.5	74
AKR thymoma, 0.4 M NaCl	2.3	51
C57BL/6 thymus, 0.4 M NaCL	2.9	0
C57BL/6 thymus, 0.25 M NaCl	3.1	0
C57BL/6 thymoma I, 0.4 M NaCl	5.4	0
C57BL/6 thymoma I, 0.25 M NaCl	9.8	0
C57BL/6 thymoma II, 0.4 M NaCl	2.1	35

* Aliquots of each enzyme in 0.02 M Tris-HCl, pH 8.0, 1 mM dithiothreitol, 0.1% NP-40, and 0.2–0.4 M NaCl were mixed with 10 μl (65 μg) of anti-Rauscher MuLV DNA polymerase or preimmune serum and incubated at 0°C for 20 min. DNA polymerase activity was measured with the preferred template (Fig. 3). One unit of DNA polymerase activity corresponds to the amount of enzyme required to incorporate 1.0 pmol of [^{3}H]TTP into acid-insoluble material for 1 h at 37°C.

‡ EU, enzyme units.

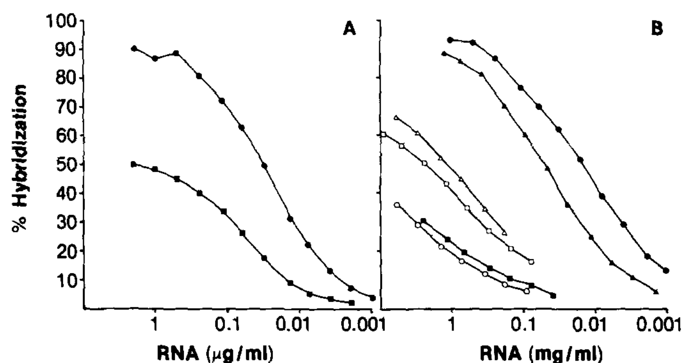

FIG. 4. Detection of virus-specific RNA sequences in leukemic and normal mice. RNA was hybridized to AKR [^{3}H]cDNA as described in Materials and Methods. (A) Hybridization of AKR MuLV cDNA with AKR MuLV 70S RNA, (●–●); and AT-124 70S RNA, (■–■). (B) Hybridization of AKR MuLV cDNA with RNA from an AKR thymoma, (●–●); from normal C57BL/6 thymus, (■–■); and from a variety of radiation-induced thymomas from C57BL/6 mice.

had relatively high concentrations of sequences hybridizing with AKR MuLV cDNA, one had a T_m of 79°C and one had a T_m of 73°C. Interestingly, both of these thymomas were characterized by the presence of an AKR MuLV type of gp71 as detected by competition assays, although the T_m and extent of hybridization of the second thymoma might suggest predominant expression of a xenotropic type virus. Nevertheless, the results illustrate that, in general, C57BL/6 thymomas can be characterized by a three- to fivefold increase in RNA sequences which hybridize with AKR MuLV cDNA and have T_m's of 72–75°C.

Data from competition and hybridization assays for several individual mice

TABLE IV
Hybridization of RNA from Normal and Leukemic C57BL/6 Tissue with AKR MuLV cDNA

Sample	Co 1/2 (mg/ml)*	Hybridization‡	T_m (°C)§
		%	
C57BL/6 thymoma	2.0	–	74
" "	2.4	–	74
" "	>12.0	–	73
" "	2.4	–	75
" "	3.2	–	ND‖
" "	6.0	–	ND
" "	3.6	–	74
" "	1.2	–	ND
" "	0.05	89	79
" "	3.6	–	74
" "	2.0	–	ND
" "	0.22	65	74
C57BL/6 thymus¶	>12.0	–	72
C57BL/6 thymus	>12.0	–	72
AKR thymoma	0.015	92	78
AKR fibroblasts** (AKR MuLV)	0.080	90	80
C57BL/6 fibroblasts** (RADLV)	0.005	88	77
AKR MuLV 70S RNA‡‡	30 (ngm/ml)	90	80
AT-124 70S RNA‡‡	100 (ngm/ml)	50	73

* Co 1/2 is the concentration of RNA required for 50% of maximum hybridization of AKR MuLV cDNA under the conditions described in Materials and Methods.

‡ Percent hybridization is the extent of hybridization at saturation of AKR MuLV cDNA. (–) indicates that saturation was not obtained and the extent could not be determined.

§ T_m is the temperature at which 50% of the hybrid is dissociated under the conditions described in Materials and Methods. The deviation for T_m values is approximately ±1°C.

‖ ND, not determined.

¶ C57BL/6 thymus RNA was obtained from control, nonirradiated C57BL/6 mice of approximately 6 mo of age.

** AKR fibroblasts were from a tissue culture cell line that spontaneously initiated replication of AKR MuLV. C57BL/6 fibroblasts were from a tissue culture cell line infected with in vivo passaged RADLV and which chronically produce the in vitro derived RADLV.

‡‡ 70S RNA was purified from AKR MuLV and the NIH Swiss xenotropic virus, AT-124, as described in Materials and Methods.

as well as the serological histories of these mice (see accompanying paper) are shown in Table V. The first two examples are of mice that died from thymomas and never developed any detectable antibody against MuLV. Thymomas from such mice consistently lacked any detectable ecotropic virus expression. Numbers 3, 4, and 8 are typical of mice that developed a transient immune response against MuLV after irradiation, but died without detectable antibodies against MuLV. Although, in general, thymomas from such mice were characterized by the lack of overt virus expression, within this group, we also obtained thymomas that had high levels of ecotropic virus expression. Most mice were comparable to numbers 5, 6, and 7, in which an immune response appeared after irradiation and persisted until death. These mice characteristically had thymomas with no detectable ecotropic virus expression.

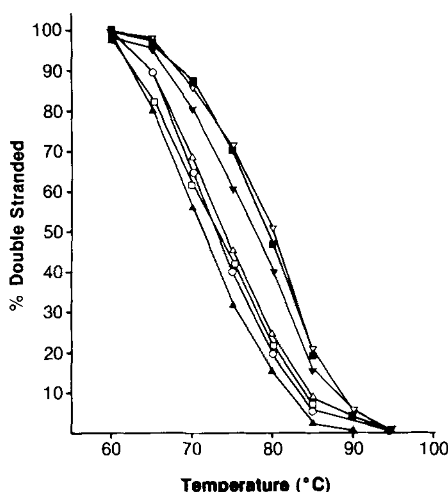

FIG. 5. Thermal stability of hybrids. DNA-RNA hybrids were formed with [^{3}H]AKR cDNA in 0.45 M NaCl at 62°C for 48 h and diluted threefold with 0.01 M Tris, pH 7.3, to contain 0.15 M NaCl. Aliquots (0.05 ml) were heated at the indicated temperature for 10 min, chilled in ice, and treated with S_1 to determine the percent double-stranded hybrid remaining. The hybrids tested were from leukemic C57BL/6 mice, (▽ – ▽, △ – △, ○ – ○); normal C57BL/6 thymus, (▲ – ▲); AT-124 70S RNA, (□ – □); AKR 70S RNA, (■ – ■); and leukemic AKR thymus (▼ – ▼).

TABLE V

Summary of Viral RNA and Antigen Expression and Serological Histories of Individual C57BL/6 Mice that Died of Radiation-Induced Lymphomas

Mouse no.*	Antibody to MuLV (months post irradiation)‡								Antibody titer at death§	Viral antigens (ng/mg protein)‖			Viral RNA¶	
	1	2	3	4	5	6	7	8		gp71	p30	p12	Co ½ (mg/ml)	T_m (°C)
1	0	0	0	0	0	0			0	<1.0	3.0	<0.2	2.0	74
2	0	0	0	0	0	0	0		0	<1.0	6.0	<0.2	>12.0	73
3	0	<1:40	<1:40	<1:40	0				0	<1.0	7.0	<0.2	3.6	74
4	0	0	0	<1:40	>1:40	>1:40	<1:40		0	95.0	16.0	8.7	0.05	79
5	0	0	<1:40	>1:40	>1:40	>1:40	>1:40		1:2,560	<1.0	6.0	<0.2	2.4	75
6	0	0	0	<1:40	>1:40	>1:40	>1:40		1:1,280	<1.0	10.0	<0.2	2.0	ND**
7	0	0	0	0	<1:40	>1:40	>1:40		1:1,280	<1.0	8.0	<0.2	1.2	ND
8	0	0	0	>1:40	<1:40				0	689	20.0	3.0	0.22	73

* C57BL/6 mice were irradiated at 1 mo of age with 175 R four times at weekly intervals.

‡ Serological histories of individual mice were derived by testing sera at monthly intervals for antibody against MuLV as described in the accompanying paper.

§ Final antibody titers were obtained from sera of moribund animals using a radioimmune precipitation assay as described in the accompanying paper.

‖ Viral antigen concentrations were determined by competition radioimmune precipitation assays as described in Materials and Methods.

¶ Viral RNA concentrations and the T_m of the hybrids were determined as described in Materials and Methods.

** ND, not determined.

Discussion

The most important conclusion from our results is that overt ecotropic virus expression is not detectable in the majority of radiation-induced thymomas of C57BL/6 mice. This conclusion is based on a variety of assays, which included

competition radioimmune precipitation assays for AKR MuLV gp71, p30, and p12; cytotoxicity assays with antisera against gp71 or p12; and assays for reverse transcriptase and hybridization of AKR MuLV cDNA with cellular RNA from thymomas. These results confirm earlier studies which failed to detect virus expression by electron microscopy (13) or by indirect immunofluorescence (14). Interestingly, infectious MuLV, which is detectable in tissue culture assays was previously shown to be absent in a number of C57BL/6 thymoma extracts (14), although these results have been interpreted to suggest that radiation-activated RADLV was defective for replication.

Although we were not able to detect several ecotropic-specific MuLV antigens in radiation-induced thymomas, the question still remains whether virus expression was below the levels of detection. In the accompanying paper we presented serological evidence which suggested that radiation activated endogenous MuLV expression, and since a number of the thymomas exmined here were from antibody-positive mice, we might have expected to find a correlation between viral antigen expression and a humoral immune response. Clearly, this was not the case; the few mice with readily detectable virus expression had only a transient immune response and lacked detectable antibody during the final stages of lymphoma development, presumably because of an excess of antigen expression. However, the development of the immune response may be a consequence of virus expression in tissues other than spleen or thymus and, in fact, irradiation of thymectomized and/or splenectomized mice results in the development of an immune response to MuLV comparable to controls (J. N. Ihle and J. Roubinian, unpublished observation). This result was not unexpected since previous experiments have indicated that activation of ecotropic virus probably occurs in bone marrow cells (13, 14). Nevertheless, even the development of an immune response to MuLV, which may be a more sensitive indication of virus expression than assays for virus antigens, was not associated with the development of lymphomas.

Also relevant to the question of levels of virus expression is the observation that in a few thymomas we found readily detectable ecotropic MuLV expression. In particular, the assays for gp71, p12, and viral RNA illustrate striking differences between the presumptive MuLV-positive and MuLV-negative thymomas. By hybridization, one of these thymomas had RNA sequences which hybridized with the majority of the AKR MuLV cDNA, and the hybrids had T_m's similar to the hybrids of AKR MuLV cDNA and AKR MuLV 70S RNA. In this particular lymphoma, a variety of techniques demonstrate the expression of the AKR type of MuLV. Our results thus suggest that there is not a spectrum of virus-positive thymomas that merge into the presumptive virus-negative thymomas, which supports the conclusion that most lymphomas are ecotropic virus negative; although as indicated in the accompanying paper, the virus was activated after irradiation.

Although our results demonstrate the lack of complete expression of MuLV in C57BL/6 thymomas, it is conceivable that all the thymomas were characterized by the expression of a leukemogenic fraction of the MuLV genome. Since AKR MuLV has been shown to be oncogenic (15), we might expect that at least the oncogenic portion would be expressed. The hybridization data suggest that this

is not the case. Specifically, in both normal and leukemic thymuses, a set of sequences with partial homology to the AKR MuLV genome is detectable. The T_m suggests that these sequences may represent expression of endogenous xenotropic viruses, which is further supported by the presence of low levels of p30 in both normal and leukemic thymuses. In most leukemic thymuses the concentration of these sequences increases approximately three- to fivefold. Nevertheless, the presence of these sequences is not unique to thymomas nor does there appear to be a small fraction of sequences which do increase significantly and uniquely. Clearly, however, this question can only be definitively examined when cDNA is made which is devoid of the naturally occurring "normal" sequences.

For many years the expression of infectious MuLV in radiation-induced C57BL/6 thymomas was supported by the ability of cell-free extracts to induce leukemia when injected in newborns and by the isolation by continued in vivo passage of such extracts of RADLV, a highly leukemogenic preparation (13, 16). Nevertheless, the correlation between either the leukemogenic factor in extracts from primary thymomas and/or in vivo passaged RADLV with endogenous ecotropic viruses is not known. Specifically, in vivo passaged RADLV is unique among most endogenous ecotropic viruses including AKR MuLV for the following reasons. (*a*) In vivo passaged RADLV is generally not readily detectable in tissue culture assays for MuLV but rather requires "blind passage" to produce detectable viral antigen expression or foci (17, 18). Once infection is established the virus is a classical infectious MuLV with serological properties identical to AKR MuLV. (*b*) In vivo derived RADLV is highly thymotropic (19), a property not associated with AKR MuLV, and appears to replicate in all dividing tissues with a preference for the spleen. (*c*) RADLV is highly thymolytic and causes thymic involution after infection (19, 20).

The difference between in vivo RADLV-induced thymic lymphomas and radiation-induced lymphomas is also striking (14, 19). Virus expression in RADLV-induced thymomas can be readily detected with indirect immunofluorescence (14, 21) and such lymphomas have p30 levels of 100-500 ng/mg protein (A. DeCleve and J. N. Ihle, unpublished observation). In contrast, the majority of radiation-induced thymomas have no virus expression detectable by indirect immunofluorescence (14) and, as shown here, generally have only low levels of p30 that are comparable to age-matched controls. Consequently, the relationship of "viruses" in radiation-induced tumors with in vivo passaged RADLV and with the tissue culture-derived ecotropic RADLV is only poorly understood.

The results demonstrated no evidence for ecotropic virus antigen expression in the majority of thymomas. These results therefore complement the serological results in the accompanying paper which demonstrated the lack of influence of an immune response on thymoma development. In particular, we have no evidence that virus-related antigens recognized by immune sera are even expressed on the majority of thymomas. The question nevertheless remains whether the immune response can either select against transformed cells expressing MuLV or actually suppress leukemia in mice in which overt MuLV expression is associated with transformation. The ability of the immune response to select against virus expression is refuted by the appearance of eco-

tropic virus-negative thymomas in antibody-negative mice. Moreover, continued transplantation of virus-negative thymomas in immunosuppressed, antibody-negative mice has thus far failed to show activation of ecotropic virus expression in these cells (J. N. Ihle and R. McEwan, unpublished observation). The ability to detect virus-positive thymomas in antibody-positive mice might actually suggest that the immune response is inefficient. Clearly, however, we can not specifically evaluate the number of times the immune response did efficiently eliminate the thymoma. In this regard, in preliminary experiments with "survivors" from radiation-induced leukemia, we have detected overt ecotropic virus expression in two of five such mice (J. N. Ihle, K. Bengali, and J. Domotor, Jr., unpublished observation). Virus expression, however, is not the only prerequisite for surviving, since some survivors have no ecotropic virus detectable and have occurred among the antibody-negative mice. Nevertheless, experiments are currently in progress to directly assess the consequences of a virus-positive tumor in an antibody-positive mouse.

Our experiments have primarily been directed at the "natural" course of events after irradiation. Of particular importance now will be to assess the effect of immunization, before irradiation, on the induction of leukemia. The results presented here and in the accompanying paper suggest that such immunizations against ecotropic MuLV would be ineffective. Nevertheless, the possibility exists that MuLV expression early after irradiation is required for induction of thymomas and if infectious spread of the virus can be stopped at this point, the animal could be protected. In this regard, it should be pointed out that in these experiments and previous experiments (22, 23) the existence of an autogenous immune response before irradiation does not provide any detectable advantage, although this point must be examined further.

The results presented here also suggest that radiation-induced leukemia in C57BL/6 mice should be an excellent experimental model for studying spontaneous leukemia in a variety of species including perhaps man, in which virus expression is not generally detected and in which there may not be a direct viral etiology. Clearly, the "spontaneous" leukemias are in contrast to naturally occurring leukemias such as those found in cats (24, 25) and cows (26, 27) in which the disease is characterized by the presence of infectious virus that is horizontally transmitted and in which there is a demonstrable seroepidemiological correlation of an immune response to the virus and leukemia. Radiation leukemia in C57BL/6 mice also appears to be quite distinct from leukemia in AKR mice, particularly with regard to the reported correlation between levels of infectious ecotropic virus and leukemia in AKR mice (28). Nevertheless, the elucidation of the molecular mechanisms involved in radiation-induced leukemia should provide an important experimental model for understanding the mechanisms involved in spontaneous leukemia in other species.

Summary

The expression of endogenous ecotropic viruses in radiation-induced thymomas of C57BL/6 mice was examined. Competition radioimmunoassays for AKR MuLV gp71, p30, and p12 were used for viral antigen expression. 3 of 40 lymphomas had readily detectable ecotropic gp71 at levels of 95–689 ng/mg

protein; the remainder of the tumors had no detectable gp71 (<1.0 ng/mg protein). 30 thymomas were characterized by the presence of MuLV p30 at levels of 1–10 ng/mg protein, levels that were comparable to those found in thymus extracts from age-matched, nonirradiated control. 10 tumors were characterized by having p30 levels of 10–30 ng/mg protein. In one tumor significant levels of AKR MuLV p12 were detectable. Since B-tropic and N-tropic viruses from C57BL/6 mice have glycoproteins (gp71) indistinguishable from AKR MuLV gp71 and the N-tropic virus had a p12 serologically identical to AKR MuLV p12, these results demonstrate that overt endogenous B-tropic virus was detectable in 2 of 40 thymomas and endogenous N-tropic virus was detectable in 1 of 40 thymomas. The lack of overt expression of gp71 or p12 was also confirmed by cytotoxicity assays using monospecific antisera to these viral proteins.

Radiation-induced lymphomas were also examined for the presence of reverse transcriptase after chromatography of tissue extracts on poly G-Sepharose. One tumor, which was characterized by the lack of gp71, also had no detectable reverse transcriptase; whereas one tumor with gp71 was characterized by readily detectable levels of reverse transcriptase in cellular extracts.

The presence of viral RNA was examined using AKR cDNA. Low levels of RNA capable of hybridizing with AKR cDNA were found in age-matched, nonirradiated mice; these hybrids had T_m's of 72°C, while hybrids with AKR MuLV 70S RNA had T_m's of 80°C. In 1 of 12 thymomas the concentration of hybridizable RNA and the T_m of the hybrids were identical to control values. In 9 of 12 thymomas the concentration of hybridizable sequences increased approximately three- to fivefold and the T_m of these hybrids varied from 73 to 75°C. In 1 of 12 thymomas the concentration of hybridizable sequences increased over 100-fold, hybridized completely with AKR MuLV cDNA, and the hybrids had T_m's of 79°C. This thymoma was also characterized by the presence of the AKR MuLV type of gp71 and p12. One tumor was characterized by a 10- to 100-fold increase in hybridizable sequences, which only partially hybridized with AKR MuLV cDNA, and hybrids had a T_m of 73°C. This tumor was characterized by the presence of AKR MuLV gp71 but not AKR MuLV p12. The results taken together demonstrate that overt endogenous ecotropic virus expression is only rarely detectable in radiation-induced thymomas of C57BL/6 mice.

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