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Changes in expression of murine leukemia virus antigens and production of xenotropic virus in the late preleukemic period in AKR mice

(thymocytes/thymus grafts/cytotoxicity tests/ecotropic virus)

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ABSTRACT **We recently reported that thymocytes from 6-month-old preleukemic AKR mice express higher levels of murine leukemia virus (MuLV)-related antigens than thymocytes from 2-month-old mice. We have now found that the level of xenotropic MuLV (defined operationally as MuLV able to infect mink cell cultures) is also markedly increased in thymus of 6-month-old AKR mice and that this increase in virus correlates closely with increased MuLV-antigen expression. There is no increase of MuLV antigen or xenotropic virus in spleen or lymph nodes. Production of ecotropic MuLV remains unchanged with age in thymus, lymph nodes, and spleen. Thymic grafts from 6-month-old AKR mice, but not from 2-month-old mice, induce both amplified MuLV-antigen expression and xenotropic virus production in the thymus of young AKR recipients. Experiments with lethally irradiated AKR mice reconstituted with syngeneic bone marrow cells indicate that age-related changes in the thymus rather than in bone marrow precursor cells are responsible for MuLV-antigen amplification.**

The critical role of the thymus in murine leukemogenesis is well known (1). In many strains of mice with a high incidence of spontaneous leukemia, the thymus is the first site of recognizable disease, and thymectomy early in life prevents its development (2). The great majority of such leukemias have T-cell markers (3), indicating that these cells have undergone some phase of their differentiation in the thymic environment. The etiology of mouse leukemia is complex, with murine leukemia virus (MuLV) (4, 5), genetic factors that limit or facilitate virus or leukemia cell replication and spread (5, 6), and humoral factors presumably elaborated by thymic stromal cells (1, 7) each playing a role.

Before the onset of leukemia, characteristic changes occur in the thymus of mice from the high-leukemia AKR strain: (*a*) the thymic cortex is reduced in size, and structures resembling lymphoid follicles and germinal centers may appear in the medulla (1); (*b*) the level of adenylate cyclase in the thymus increases (8); and (*c*) the thymocyte undergoes a marked change in the expression of certain cell surface antigens (9, 10). We have reported recently that starting at about 4–5 months, MuLV-related antigens (G_{IX}, GCSA, p30, and gp70) and H-2 alloantigens on thymocytes show a progressive increase, whereas the differentiation alloantigen Thy-1 is reduced (9).

The present study is a further analysis of the preleukemic changes occurring in AKR mice, with the following questions being asked. (*i*) Is the increase in MuLV-antigen expression associated with an increased level of MuLV infectivity? (*ii*) Is the increase in MuLV-antigen expression restricted to the thymus, or do other lymphoid organs undergo similar changes? (*iii*) Can amplification of MuLV-antigen expression be induced by thymic grafts from preleukemic mice? (*iv*) Are the preleukemic thymic changes due to age-related changes in bone marrow precursor cells or to altered properties of the thymic environment?

MATERIALS AND METHODS

Mice. Mice were from our colony at the Sloan-Kettering Institute or from The Jackson Laboratory, Bar Harbor, Me.

Cytotoxic Test (11). Equal volumes (50 μl) of serially diluted antiserum, cells (5×10^6/ml), and complement (guinea pig serum diluted 1:4) were mixed and incubated at 37° for 45 min. Viability counts were made in the presence of trypan blue.

Immunofluorescence Absorption Test. The method of Hilgers *et al.* (12) was used with slight modifications. A 20% extract of the tissues to be tested was made in 0.5% Nonidet P-40 in phosphate-buffered saline. Absorption tests were performed with a concentration of antiserum two doubling dilutions below the endpoint on acetone-fixed EδG2 [a transplanted C57BL/6 leukemia induced by passage A Gross virus (13)]. Serially diluted tissue extract (40 μl) was mixed with an equal volume of diluted antiserum and incubated overnight at 4°. The mixture was tested for residual immunofluorescence activity on acetone-fixed EδG2 cells. Antigen titers in Fig. 1 are expressed as the highest dilution of extract capable of absorbing immunofluorescence activity.

Antisera. For cytotoxic tests: (W/Fu × BN)F_1 anti-W/Fu leukemia (C58NT)D (= anti-NTD) (11, 14). For IFA tests: goat anti-MuLV-gp70 (Rauscher) (15) provided by M. Strand and J. T. August, Albert Einstein College of Medicine, and rabbit anti-MuLV-p30 (Rauscher) (16) provided by E. Fleissner and W. D. Hardy, Jr., of this Institute. For fluorescent antibody focus assays on infected tissue culture cells: fluorescein isothiocyanate-conjugated goat antiserum prepared against Moloney MuLV disrupted by Tween–ether (lot no. 5010101, prepared by R. E. Wilsnack and supplied by the National Cancer Institute).

Infectious Center Assays in Tissue Culture. Suspensions of lymphoid cells were prepared by mincing thymus and lymph node tissue with a scalpel and by forcing saline through the spleen with a syringe and 25-gauge needle. The cells were washed, counted, and diluted to a concentration of 1 to 2×10^7/ml. They were exposed to 25 μg/ml of mitomycin C for 30 min at 37°, washed twice, and resuspended to 10^7 cells per

Abbreviations: MuLV, murine leukemia virus; anti-NTD, (W/Fu × BN)F_1 anti-W/Fu leukemia (C58NT)D antiserum.

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ml in 10% heated (56°, 30 min) fetal calf serum in Dulbecco's modified basal medium (10-DMB). Appropriate serial dilutions were plated on indicator cell cultures, essentially as outlined by Melief *et al.* (17). Indicator cultures had been plated at 2×10^5 cells per 60 mm diameter petri dish the previous day. Medium was changed on the day following infection and at 2- to 4-day intervals thereafter.

The indicator cell for detection of ecotropic virus was the wild mouse embryo cell line SC-1, grown and maintained as previously described (18). Dilutions of lymphoid cells were added to cultures containing Polybrene (16 μg/ml). Virus was detected by the XC plaque test (19) performed on the sixth day.

Xenotropic virus was detected by plating on the mink lung cell line CCl-64 (20), grown and maintained in 10-DMB and pretreated with DEAE-dextran (19). Lymphoid cells were titrated in cultures containing coverslips; these were maintained in medium containing unheated serum. Coverslips were fixed on the fifth day and stained, and the immunofluorescent foci were counted, as previously described (21). In those cases where no staining was seen, the cultures were transferred at 10–14 days onto fresh coverslips and examined by immunofluorescence about 5 days later.

Mink cultures with large numbers of immunofluorescent loci induced by AKR thymocytes did not produce significant amounts of XC-positive ecotropic virus, but did contain virus infectious for mink cells. For the purpose of this report, viruses capable of infecting mink lung cells are designated as xenotropic, although preliminary data suggest that a number of such isolates from AKR mice possess characteristics that may set them apart from previously reported xenotropic MuLVs. AKR thymus cells that were strongly positive by immunofluorescent focus induction in mink lung cells did not register as efficient infectious centers in simultaneous tests in the "sarcoma-positive, leukemia-negative" (S^+L^-) mink lung cell line (22).

Tissue Grafts. Fragments of thymus, spleen, or lymph node (approximately 2 mm^3) were prepared by mincing tissue dissected sterilely from donor mice. Each recipient received two to three fragments placed under the kidney capsule with fine forceps or subcutaneously by trocar. The two methods of grafting gave identical results.

Thymic Biopsies. These were performed according to the method of Boyse *et al.* (23).

Bone Marrow Reconstitution of Lethally Irradiated Mice. Seven days following thymic biopsy, AKR mice were exposed to 800 R (0.2 C/kg) whole body irradiation (General Electric Maxitron). Approximately 4 hr later, each mouse was injected with 2 to 4×10^7 bone marrow cells via the retroorbital plexus. The bone marrow cells were prepared from femur and tibia of individual AKR donors whose thymocytes were tested for anti-NTD reactivity. Each irradiated recipient received bone marrow cells from a single donor.

RESULTS

Comparison of MuLV-Antigen Expression in Thymus, Spleen, Lymph Nodes, and Uterus of 2- and 6-Month-Old AKR Mice. In our previous report (9), the increased MuLV-antigen expression of 6-month-old AKR thymocytes was demonstrated in three ways: (*i*) direct cytotoxic tests with the broadly reactive MuLV antiserum anti-NTD, (*ii*) quantitative absorption of cytotoxic antibody to MuLV-related cell surface antigens G_{IX} and GCSA, and (*iii*) membrane immunofluorescence tests with antisera to MuLV-structural antigens p30 and gp70.

In the present study, MuLV-p30 and gp70 levels in thymus, spleen, and uterus of 2- and 6-month-old AKR mice were determined by quantitative immunofluorescence absorption tests. As shown in Fig. 1, these antigens increase sharply in amount in the 6-month-old AKR thymus, confirming our previous observations. Fig. 1 also shows that the increase is specific for thymus; titers of MuLV antigens in spleen and uterus are initially higher than in thymus, but undergo little or no change with age. Leukemic thymus tissues have levels of MuLV antigen that are comparable to 6-month-old thymus.

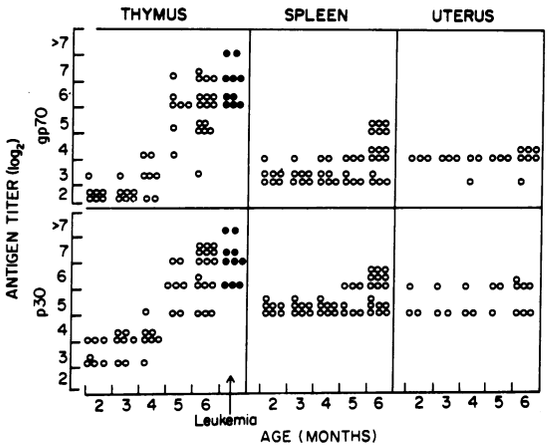

FIG. 1. Quantitation of MuLV-p30 and gp70 antigens by immunofluorescence absorption tests in thymus, spleen, and uterus of AKR mice, aged 2–6 months. Circles represent tests with tissues from individual donors.

Despite the presence of p30 and gp70 antigens in peripheral lymphoid tissue, there was no detectable cytotoxicity of anti-NTD with cells from spleen or lymph nodes at either 2 or 6 months. This could reflect a difference between thymocytes and peripheral lymphocytes in sensitivity to anti-NTD cytotoxicity, or could merely indicate that MuLV-antigen-positive cells are a minority component of the more heterogeneous peripheral lymphoid cell populations.

Assays for Ecotropic and Xenotropic MuLV. No difference was observed in the number of cells producing ecotropic MuLV in thymus of 2- or 6-month-old AKR mice (Fig. 2). The number of cells producing xenotropic virus, however, increased markedly between the second and sixth month; the 2-month thymuses were negative or showed only a rare positive cell, while 6-month thymuses (with one exception) contained large

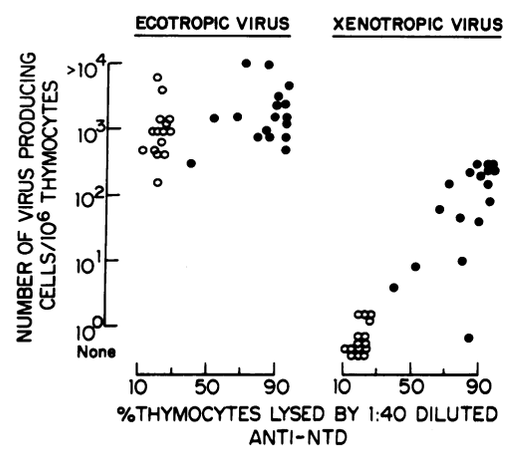

FIG. 2. Thymocytes of individual AKR mice, aged 2 months (○) or 6 months (●), tested for anti-NTD sensitivity and for production of ecotropic and xenotropic virus.

Table 1. Tests for MuLV-related antigens and for ecotropic and xenotropic viruses in tissues of 2- and 6-month-old AKR mice

Age of AKR donors	% Thymocytes lysed*	Virus assays† [infectious centers (log_{10}) per 10^6 cells, ecotropic/xenotropic] Thymus	Spleen	Lymph nodes
2 Months	12	2.7/tr	3.8/0.4	
	17	2.7/–	3.9/0.4	
	19 }	3.2/0.9	3.7/tr	3.6/–
	21 }			3.6/–
	20	3.0/–	3.5/–	3.6/tr
	22	3.1/tr	3.5/–	3.7/tr
6 Months	66	3.2/1.8	3.9/tr	
	78	2.9/1.7	3.7/tr	3.7/tr
	84	2.9/–	3.7/tr	3.7/tr
	88	3.2/2.5	3.7/tr	3.7/tr
	89	3.4/1.6	3.7/0.4	
	90	3.5/2.3	3.6/0.5	
	91 }	3.0/1.4	3.9/tr	3.8/0.2
	95 }			3.5/tr
Leukemic‡		3.8/~2.5		
		4.0/>3.0		

* Cytotoxic tests with anti-NTD serum diluted 1:40.

† Blank space indicates not tested. Brackets indicate that the thymuses were pooled for virus testing. – = negative on initial assay and blind passage; tr = trace, meaning negative on initial assay but a few immunofluorescent foci seen on blind passage of the assay culture. The data on the 2- and 6-month-old thymuses are included in Fig. 2.

‡ AKR leukemias uniformly show ≥95% cytotoxicity with anti-NTD serum.

numbers of positive cells. Further, tests of thymocytes from individual AKR mice revealed a close correspondence between the level of xenotropic virus production and MuLV-antigen expression (Fig. 2).

Ecotropic virus production by lymph node and spleen cells was similar in 2- and 6-month-old AKR mice (Table 1). In contrast to the preleukemic increase in xenotropic virus in thymus, very few cells in the spleen and lymph nodes produced xenotropic virus at either age.

Influence of Thymic Grafts on MuLV-Antigen Expression. Two-month-old AKR recipients received thymus grafts from 2- or 6-month-old syngeneic donors. Fig. 3 shows cytotoxic tests with cells from the recipient's thymus 4–5 weeks after grafting. Expression of MuLV-related antigens on thymocytes was not influenced by thymus grafts from 2-month-old donors, but was amplified by grafts from 6-month-old donors. Grafts of lymph nodes or spleen from 6-month-old AKR donors failed to increase MuLV-antigen expression on thymocytes (data not shown).

To exclude the possibility that cells from the thymic graft simply repopulate the recipient's thymus, experiments were performed with AKR-H-2^b mice (24) as donors. AKR-H-2^b mice, which differ from AKR at the *H-2* locus, undergo the same preleukemic change as AKR in MuLV-antigen expression on thymocytes. Grafts of 6-month-old AKR-H-2^b thymus also induced amplified expression of MuLV-antigens in the thymus of 2-month-old AKR mice. Thymocytes from such grafted animals continued to express H-2^k antigen characteristic of the AKR recipient, rather than H-2^b of the thymic graft donor.

Influence of Thymic Grafts on MuLV Production. The number of cells producing xenotropic virus was markedly increased in the thymus of 2-month-old recipients receiving

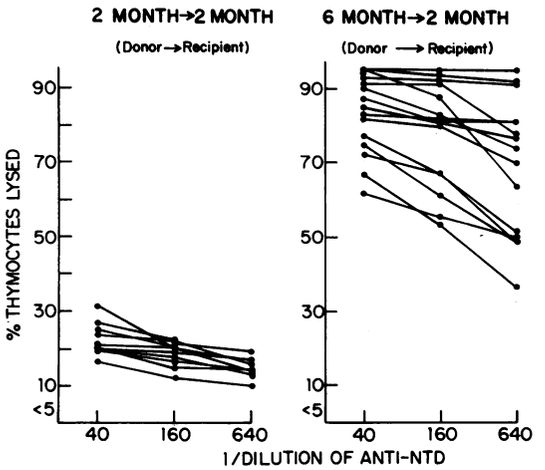

FIG. 3. Cytotoxicity tests with thymocytes of young AKR recipients grafted 4–5 weeks previously with thymus from a single 2- or 6-month-old AKR donor. (The 6-month-old donors were selected on the basis of thymocyte cytotoxicity with anti-NTD serum; only those with amplified MuLV expression were used as a source of thymus tissue.) Each line represents a titration with thymocytes from a single recipient.

thymic grafts from 6-month-old donors (Fig. 4) but not from 2-month-old donors. Production of ecotropic virus in the recipient thymus was not affected. Once again, there was a correspondence between the level of xenotropic virus and MuLV-antigen expression in tests on individual thymuses.

MuLV-Antigen Expression in Thymus of Lethally Irradiated AKR Mice Repopulated with Syngeneic Bone Marrow. Two- and six-month-old AKR mice, 1 week after thymic biopsy, were irradiated with 800 R followed by 2 to 4 × 10^7 bone marrow cells from 2- or 6-month-old AKR donors. Irradiated AKR mice not receiving bone marrow cells died within 8–12 days, and mice reconstituted with allogeneic bone marrow cells (CBA or C57BL-H-2^k) showed replacement of thymus by cells marked by donor Thy-1.2 alloantigen.

Fig. 5 shows cytotoxic tests with thymocytes of the repopulated recipients 3–4 weeks after irradiation. Thymocytes from 2-month-old recipients receiving 2-month-old bone marrow had the expected low MuLV-antigen expression, and 6-month-old recipients receiving 6-month-old bone marrow had

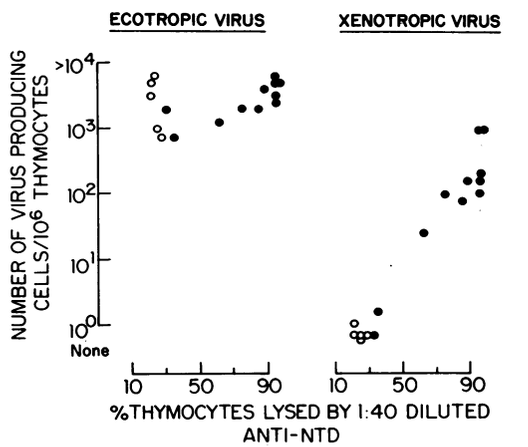

FIG. 4. Two-month-old AKR mice were given thymus grafts from 2-month (○) or 6-month (●) -old AKR donors. Four to 5 weeks later recipient thymocytes were tested for anti-NTD sensitivity and for production of ecotropic and xenotropic virus. For values in mice not receiving grafts, see Fig. 2 and Table 1.

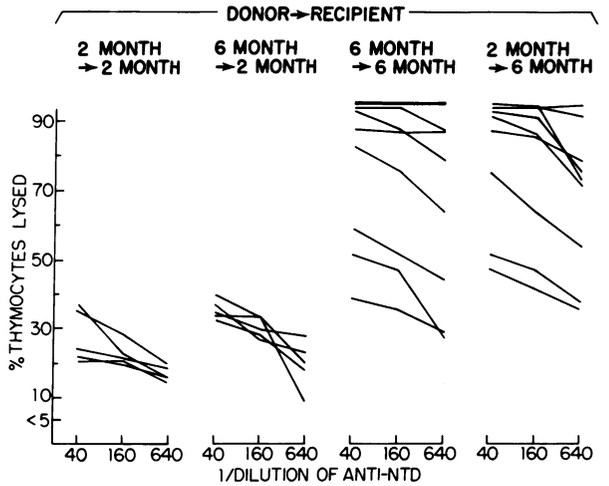

FIG. 5. Cytotoxicity tests with thymocytes of lethally irradiated AKR mice reconstituted with bone marrow cells from 2- or 6-month-old AKR mice. Day 0: thymus biopsy of AKR recipient. Thymocytes tested with anti-NTD serum. Day 7: irradiation with 800 R. Three to 4 hr later, 2 to 4 × 10^7 bone marrow cells were injected intravenously. Day 28–35: cytotoxicity tests with thymocytes from irradiated recipients; the results are shown in this figure. Each line represents a titration with cells from a single donor.

the expected high expression of MuLV antigens. Thymocytes from 2-month-old mice receiving 6-month-old bone marrow (from donors with amplified thymic MuLV expression) showed sensitivity to anti-NTD identical to that of normal, nonirradiated 2-month-old mice. AKR mice aged 6 months reconstituted with cells from 2-month-old donors retained the characteristically amplified phenotype of 6-month-old thymocytes. Thus, the preleukemic amplification in MuLV-antigen expression on thymocytes appears to be due to age-related changes in the thymus rather than to changes in the bone marrow precursor cell population.

DISCUSSION

It has long been known that morphologic changes occur in the preleukemic AKR thymus which are not seen in other lymphoid tissues (1). The preleukemic changes in MuLV antigen and virus expression that have now been recognized also appear to be restricted to the primary site of leukemia development—the thymus. As ecotropic virus is generally thought to be the leukemogenic virus of AKR mice, it might be expected that this virus would increase during the preleukemic period and account for the amplified expression of MuLV-related antigens in thymus and for the accelerating effect of cell-free extracts of leukemic and preleukemic thymus on leukemia development in AKR mice (25–27). However, no difference in the level of ecotropic virus in 2- and 6-month-old thymus was observed in this study using infectious center assays, or in past studies with titrations of tissue extracts (28). Xenotropic virus, on the other hand, has not as yet been shown to have oncogenic activity in mice or rats, and it remains to be seen whether the increased production of xenotropic virus occurring in preleukemic thymus signifies a critical role for this virus in AKR leukemogenesis.

Which viral genome(s) is coding for the increased expression of MuLV antigens in 6-month-old thymus is not clear. The close correlation between MuLV antigens and level of xenotropic virus in preleukemic thymus certainly suggests that increased production of xenotropic virus is involved; on the other hand, more cells are producing ecotropic virus at 2 and 6 months than are detected as xenotropic virus-producers at their peak. Since some MuLV antigens show type specificity, it might be possible to infer the nature of the responsible genome from the pattern of antigens involved. However, under the conditions of testing used here, group reactive determinants of MuLV-p30 and gp70 are detected; it is not yet known to what extent GCSA and G_{IX} typing may be able to distinguish between ecotropic and xenotropic viruses. In recent studies, four xenotropic virus isolates from non-AKR sources failed to induce G_{IX} antigen in infected cells (29); testing of isolates of xenotropic virus from AKR mice for G_{IX}-inducing capacity is not yet completed.

Grafts of 6-month-old thymus exhibiting amplified MuLV-antigen expression induced similar amplification in the thymus of 2-month-old recipients. This change in the recipient's thymus is not due to thymic repopulation by donor thymocytes, since in experiments with AKR-H-2^b grafts to young AKR (H-2^k) mice, thymocytes with amplified MuLV expression showed no detectable H-2^b alloantigen. Although we cannot exclude transient presence of cells from the thymus graft in the host thymus, studies with chromosomally marked thymocytes have shown that these cells do not have the capacity to repopulate the thymus (30). Two factors that might account for the effect of the grafted thymus are xenotropic virus or augmented production of a thymic hormonal factor. The grafts need act for only a limited period; the amplified state persists following surgical removal of the thymic graft (data not shown here) or rejection of the thymic graft (in the case of AKR-H-2^b donors). If a xenotropic virus is involved, it must be one that has acquired the ability to infect mouse cells, at least cells of the mouse thymus. A number of the MuLV strains isolated in mink cells in the present study have in fact induced antigen in mouse cells (J. W. Hartley and W. P. Rowe, unpublished data), which could represent recombination or phenotypic mixing with ecotropic virus.

When mice are lethally irradiated and injected with syngeneic bone marrow, the reconstituted thymus is known to be derived from two sources—thymocytes from precursor cells present in the donor bone marrow, and thymic stromal cells (which are radiation resistant) from the original recipient. This has permitted us to analyze which cell population is responsible for the age-related amplification in MuLV-antigen expression that occurs in AKR thymus. As the amplified phenotype is determined by the age of the recipient and not by the age of the bone marrow donor, attention is focused on changes in the properties of the stromal cells. The importance of the thymic stroma in leukemia development has been emphasized by many investigators (1, 7, 26). With regard to the AKR strain, Law reported (31) that grafts of AKR thymus (from 1-month-old donors; L. W. Law, personal communication, 1976) increased the incidence of leukemia in (C3H × AKR)F_1 recipients; grafts of thymus from the low leukemia incidence C3H strain did not. Such thymic grafts rapidly become chimeric, with thymocytes derived from F_1 cells and thymic stroma from the graft donor. As a consequence, the leukemias that resulted in F_1 mice grafted with AKR thymus were of F_1 origin, as determined by transplantation tests. Law's experiment points to the fact that AKR, but not C3H, stromal cells provide the leukemogenic signal, whether virus, humoral, or otherwise.

The morphological changes in the preleukemic AKR thymus, which include depletion of cortical thymocytes and the appearance of structures resembling lymphoid follicles in the thymic medulla, have suggested to Metcalf (1) that the preleukemic thymus may be the site of an autoimmune reaction which, in some way, initiates the leukemogenic process. The finding of immunoglobulin with demonstrable MuLV reac-

tivity in glomeruli of AKR kidney (32, 33), the fact that leukemia cells are selectively destroyed when AKR mice are infused with complement (34), and the presence of low levels of viral antibody in AKR serum (35, 36) are consistent with the possibility that AKR mice may be undergoing an immunological reaction to antigens of thymic origin, present on both preleukemic and leukemic thymocytes. A possible source for these antigens, and one to which the AKR mouse might not be tolerant, is the xenotropic virus that we now know appears in the thymus in later life. Search for naturally occurring cellular and humoral immunity to AKR xenotropic virus and the antigens it induces may well be rewarding.

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