AGE-RELATED CHANGES IN CELL SURFACE ANTIGENS OF
PRELEUKEMIC AKR THYMOCYTES*

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Much of the vast amount of research that has been done on leukemogenesis in
the mouse has centered on the AKR strain.

In this inbred strain, which Furth developed by selecting for high incidence of leuke-
mia, the majority of mice die of leukemia within 1 yr (1). These leukemias are virtually
all of the T-lymphocyte type and originate in the thymus or from cells derived from the
thymus. The incidence of leukemia is greatly reduced by thymectomy in early life (2). It
is from this strain that Gross (3) first isolated murine leukemia virus (MuLV),† and AKR
mice are now known to carry at least two chromosomal loci, demonstrated by Rowe and
his colleagues to be integrated MuLV genomes which independently entail the spontane-
ous production of MuLV in high titer from around the time of birth (4). Other loci that
predispose to leukemia are (a) Fv-1, which governs infective dissemination of MuLV (5)
and (b) H-2-linked genes of which one may control proliferation of MuLV and MuLV-
infected cells via immune responsiveness (6, 7), and another which may concern the pro-
duction of virions (8).

Leukemia in AKR mice is obviously related to age, the incidence rising
sharply between 6 and 12 mo after birth. This has not been accounted for in
terms of MuLV production, which reaches a plateau in very early life and does
not greatly increase until the onset of overt leukemia (9). Much the same has
been reported of quantitative measurements of viral gs antigens (10). Nonethe-
less, there is ample evidence of morphological thymic changes, such as cortical
atrophy, which precede the development of leukemia (11).

In our work with MuLV-associated antigens (12), we have been impressed by
their apparently far greater expression on leukemia cells as compared with
MuLV-infected but nontransformed lymphoid cells. We assumed to begin with
that this 'amplification' was synchronous with the leukemogenic transformation
itself. But this we find is not the case, as is shown in the following report on
MuLV-associated and other surface antigens expressed on thymocytes of AKR
mice at various ages.

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† Abbreviations used in this paper: anti-NTD, (W/Fu × BN)F1 anti-W/Fu(C58NT)D; B6, C57BL/ 6; GCSA, Gross cell surface antigen; MuLV, murine leukemia virus.

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Materials and Methods

Mice. From our colonies or from The Jackson Laboratory, Bar Harbor, Maine. For description of congenic strains see Table I.

Antisera, Reference Cells, and Complement Source. See Table II.

Cytotoxic Test (16). Equal volumes of cells (5 x 10^6/ml), a serial dilution of antiserum and an appropriate dilution of complement were mixed and incubated at 37°C for 45 min. After addition of 0.16% freshly prepared trypan blue, a count of stained and unstained cells was made.

Quantitative Absorption Test (22, and Table II). After absorbing aliquots of diluted antiserum with a range of counted numbers of cells, the residual activity of the serum was tested in the standard cytotoxic test.

Absorption Index. (a) Gx and Gross cell surface antigen (GCSA): the number of cells (B6-Gix + thymocytes from 2-to-old donors for Gx assays and pooled spleen cells from 2 to 3-to-old AKR mice for GCSA assays) required to reduce the cytotoxicity of reference antiserum to 40% lysis of the standard test cell divided by the number of AKR thymocytes or leukemia cells required to reduce the cytotoxicity of the antiserum to the same level. (b) H-2, Thy-1, and Ly-2: the number of cells from 2-to-old donors (10 x 10^6 B6-H-2a thymocytes for H-2a, 3 x 10^6 A-Thy-1.1 thymocytes for Thy-1.1 assays, and 3 x 10^6 B6-Ly-2.1 thymocytes for Ly-2.1 assays) divided by the number of AKR thymocytes or leukemia cells which give the same percent lysis of the standard test cell.

Membrane Immunofluorescence Test for gp70 and p30 Antigens (23). A mixture of 1:40 diluted antiserum (50 fi) and cell suspension (25 fi, 2 x 10^7/ml) was incubated at 4°C for 30 min. The cells were washed twice and resuspended in 50 fi fluoresceinated antigoat or rabbit immunoglobulin depending on the source of the antiserum. After incubation for 30 min at 4°C, the cells were rewashed twice and examined with a Leitz Orthoplan microscope equipped with an Osram HBO 200 mercury lamp, and BC 38 exciter, KP 490 interference, and K 530 barrier filters.

The two antisera used in these tests were: goat anti-gp69/71 MuLV-Rauscher (24), provided by Doctors M. Strand and J. T. August, Albert Einstein College of Medicine, and rabbit anti-p30 MuLV-Rauscher (25), provided by Doctors E. Fleissner and W. D. Hardy, Jr., of this Institute.

Bovine Serum Albumin (BSA) Gradient Separation (26). Approximately 10^6 AKR thymocytes were suspended in 1 ml of 35% BSA (Path-o-cyte 5, Pentex, Miles Laboratories Inc., Kankakee, Ill.) in a 5-ml Beckman cellulose-nitrate centrifuge tube. On this was layered 1 ml each of 26, 23, 20, and 10% BSA (diluted in medium 199), and the tube was then centrifuged at 4°C at 13,000 rpm for 30 min in a Beckman SW 50.1 rotor. The four layers obtained were designated A (at the 10-20% interface), B(20-23%), C(23-26%), and D(26-35%).

Cytograf Analysis. AKR thymocyte suspensions were adjusted to 2 x 10^6 cells per ml and examined with a Cytograf Model 6300 (Bio/Physics Systems Inc., New York), coupled with a pulse height analyzer (NS Econ Series, Northern Scientific Inc., Wisconsin).

Results

Cytotoxic Tests with Broadly Reactive MuLV Antisera. Thymocytes from AKR mice of various ages were examined in direct cytotoxic tests with (W/Fu x BN)F1 anti-W/Fu(C58NT)D serum. This antiserum (which is referred to as anti-NTD) was produced by immunization with a MuLV-induced transplantable lymphoma of W/Fu origin and contains antibodies to a spectrum of MuLV-related antigens (15). Fig. 1 illustrates cytotoxic tests with thymocytes from individual 2 wk to 6-to-old AKR mice and with thymic leukemia cells. Thymocytes from young AKR mice show a low level of cytotoxicity with anti-NTD serum, whereas thymic leukemia cells are strongly reactive. Cytotoxic tests with thymocytes from individual 6-to-old mice reveal that anti-NTD reactivity falls into two categories, low sensitivity comparable to young mice and high sensitivity approaching that seen with AKR leukemia cells.

Characteristics of Thymocytes from Preleukemic Mice. Before detailed analysis of the amplified expression of MuLV antigens in the thymus of 6-to-old AKR mice, it was essential to exclude the possibility of microscopic leukemia
cell infiltration and replacement. As a rule, thymic leukemia cells are clearly larger than normal thymocytes, and the experienced microscopist has little difficulty in distinguishing them. Such inspection revealed no significant difference in size between thymocytes from 2-mo and 6-mo-old AKR mice. Examination of cell size distribution by Cytograf analysis substantiated this. In addition, separation of cells by BSA gradient centrifugation showed essentially the same distribution pattern for thymocytes from 2-mo and 6-mo-old mice. The predominant cell type of the thymus, the small cortical thymocyte, is found in the D layer; thymocytes from 6-mo-old AKR mice showing increased anti-NTD sensitivity were also found in this layer.

The critical test for the presence of leukemia cells is transplantability to syngeneic hosts. 5-10 × 10^6 leukemia cells from AKR mice with thymoma produces evident disease in young AKR recipients within 3 wk; leukemia did not result (observation period 100 days) from the inoculation of 50-100 × 10^6 thymo-
Cells from individual 6-mo-old AKR mice with amplified MuLV antigen expression.

Age-Related Changes in the Quantity of Individual MuLV-Related Antigens Expressed on AKR Thymocytes. As anti-NTD is a polyvalent antiserum capable of detecting a range of viral and cellular antigens related to MuLV, we set about to determine whether the increased reactivity of preleukemic thymocytes to anti-NTD is due to increased coordinate expression of all MuLV antigens or to selected determinants only. For this reason, the amount of four MuLV-related antigens, G\textsubscript{IN}, GCSA, gp70, and p30, on AKR thymocytes was determined.
GiX Antigen

GiX was first detected on normal thymocytes of the low leukemia incidence strain 129 in cytotoxic tests with anti-NTD (16). In typing thymocytes of mice from various strains, two GiX phenotypes could be distinguished, GiX- and GiX+. The GiX+ group could be further subdivided on the basis of quantitative expression of GiX into GiX1, GiX2, GiX3 strains. AKR belongs to the GiX2 strains; thymocytes of GiX2 mice express about two-thirds the quantity of GiX compared to GiX3 mice (129, B6-GiX+, etc.). GiX- cells can be converted to GiX+ by MuLV infection in mice as well as rats, suggesting coding of GiX by viral genes rather than host genes (16). It was originally thought that GiX was a nonvirion antigen specified by MuLV, since it occurred on thymocytes of strains such as 129 that showed no evidence of MuLV infection. We now know that GiX is a type-specific determinant of the major envelope glycoprotein of MuLV, gp70, which can be independently expressed without concomitant expression of other viral gene products (27, 28).

Fig. 2 illustrates individual quantitative absorption tests for GiX, and the results of all tests are summarized in Fig. 3. Thymocytes from the low leukemic B6-GiX+ strain served as control cells (see Table II). AKR thymocytes reach adult GiX2 levels at about 6 wk of age, and this does not change for several months. At 5-6 mo, the majority of AKR mice showed a threefold or greater increase in the amount of GiX antigen. Leukemia cells invariably express the highest amount of GiX. In contrast to AKR mice, expression of GiX antigens on thymocytes of individual B6-GiX+ mice remains constant with age. Two other GiX+ strains with low leukemia incidence, 129 and A, show no age-related change in the level of GiX antigen on thymocytes.

GCSA Antigen

GCSA is found on the surface of cells replicating MuLV and, as such, is a useful marker for overt MuLV infection (29). All mouse strains with a high incidence of leukemia (AKR, C58, AKR-H-2b, C3H/Fl, PL) type GCSA+; low incidence strains are either GCSA- or show levels of the antigen below the level of sensitivity of the GCSA typing method (17). Aging is known to influence expression of GCSA; spleens from young C3H/FlBi are GCSA-, but by 6 mo of age they convert to GCSA+ (30). As GCSA typing sera failed to react with the viral envelope in immunomicroscopy, it was concluded that GCSA, although virus specified, was not a structural component of MuLV (31). Further work is needed to clarify this question, since GCSA may still be a component of the virus, but occupy a position in the intact virion that is inaccessible to antibody.

Fig. 4 illustrates individual quantitative absorption tests for GCSA, and the results of all tests are summarized in Fig. 5. In young AKR mice, thymocytes show far lower levels of GCSA than spleen cells (17). By 6 mo, however, the quantity of GCSA on AKR thymocytes is generally greatly increased. This increase in GCSA on 6-mo thymocytes corresponds to the high GCSA levels found on AKR leukemic cells.

gp70 and p30 Antigens

Eight polypeptides, including reverse transcriptase, have been identified in MuLV (25, 32). The two structural components most intensively studied are gp70, the major glycoprotein of the viral envelope, and p30, the major core protein. Although it was expected that gp70 would be expressed on the cell surface, the demonstration that p30 determinants could also be detected on the surface of cells came as a surprise (33). Whether p30 is an integral part of the membrane or simply adsorbed to the surface is currently being studied.
Fig. 2. Estimation of the amount of $G_{ix}$ antigen by quantitative absorption analysis. Bold figures (under arrow) refer to the number of thymocytes or thymic leukemia cells from individual donors required to reduce the cytotoxic activity of anti-NTD serum to 40% lysis of reference $G_{ix}^+$ test cells ($B6-G_{ix}^+$ thymocytes from 2-mo-old donors). Four separate experiments are illustrated; comparison of absolute cell numbers required for absorption is valid only within individual experiments.

Fig. 6 summarizes our data on surface gp70 and p30 of AKR thymocytes and leukemia cells detected by indirect membrane immunofluorescence tests. Thymocytes of AKR mice aged 1–5 mo show virtually no reactivity with anti-gp70 or anti-p30. By 6 mo, increased numbers of p30$^+$ and gp70$^+$ cells appear in the thymus, and there is a strong tendency for coordinate expression of these two antigens in individual mice. Leukemia cells exhibit strong gp70 and p30 reactivity. Immunofluorescence tests on sections of 6-mo-old AKR thymus localize increased gp70 and p30 expression to the thymic cortex.
Fig. 3. Quantitative differences expressed as an absorption index in the amount of $G_{ix}$ antigen on thymocytes from AKR and B6-$G_{ix}^+$ mice of various ages and on AKR thymic leukemia cells. Each symbol represents results obtained with cells from a single donor. Reference cells for calculating absorption index; B6-$G_{ix}^+$ thymocytes from 2-mo-old donors.

Fig. 4. Estimation of the amount of GCSA by quantitative absorption analysis. Bold figures (under arrow) refer to the number of cells from individual donors required to reduce the cytotoxic activity of B6 anti-K36 serum to 40% lysis of reference GCSA+ test cells (B6 MuLV-induced leukemia, E $\div$ G2). Two separate experiments are illustrated; comparison of absolute cell numbers required for absorption is valid only within individual experiments.
Fig. 5. Quantitative differences expressed as an absorption index in the amount of GCSA on thymocytes from AKR mice of various ages and on AKR thymic leukemia cells. Each symbol represents results obtained with cells from a single donor. Reference cells for calculating absorption index; GCSA+ spleen cells from 2-3-mo-old AKR donors.

Fig. 6. Membrane immunofluorescence tests for gp70 and p30 antigens on thymocytes from AKR mice of various ages and on thymic leukemia cells. A line connects results for individual mice.
Age-Related Changes in the Expression of H-2, Thy-1, and Ly-2 Alloantigens on AKR Thymocytes

Thymocytes from adult mice characteristically express little H-2 and much Thy-1 and Ly alloantigens. This contrasts with the high H-2/low Thy-1 and Ly phenotypes of peripheral T lymphocytes. Alloantigens specified by the H-2 locus are found on a variety of cell types. Thy-1 and Ly antigens have a more restricted tissue distribution, with antigens of the Ly series being found only on cells of T lineage (34).

Fig. 7–11 summarize our studies on the expression of H-2, Thy-1, and Ly-2 antigens on thymocytes of AKR mice of various ages and on thymic leukemia cells. Direct cytotoxic tests and quantitative absorption analysis reveal that thymocytes from 6-mo-old mice may have two to three times the quantity of H-2 antigens expressed by thymocytes of younger mice. This high H-2 level of thymocytes from preleukemic mice parallels the high H-2 phenotype of thymic leukemia cells. Age-related changes in Thy-1 antigen follow a reverse pattern. Thy-1 expression is highest on 2-mo-old AKR thymocytes and falls with age. Leukemia cells, like 6-mo thymocytes, show a low Thy-1 phenotype. Although not as striking as changes in Thy-1 levels, the quantity of Ly-2 also decreases with age.

Thymocytes from mouse strains with a low incidence of leukemia do not show significant age-related changes in the expression of H-2, Thy-1, or Ly-2 antigens (Fig. 9–11). In addition to the strains reported in this study, we have examined the level of H-2 and Thy-1 antigens on thymocytes from 2 and 6-mo-old mice of a variety of other low incidence strains, including C3H/An, B6, B6-Gx+, 129, A, and DBA/2 mice. In no instance did we find thymic changes comparable to those observed in AKR mice.

Discussion

From the time that Willis enunciated his 'field therapy' of cancer (35), it has become increasingly clear that cancer is not characteristically initiated as an abrupt event in which a normal cell acquires autonomy. Foulds coined the term 'tumor progression' in reference to the sequential deviations from normality which precede the evolution of frank malignancy in the mouse mammary gland (36). Tumor progression is perhaps most exquisitely illustrated by endocrine tumors of rodents (37). Here the progress to autonomy, defined as transplantability to nonconditioned hosts, is preceded by stages described as 'hormone-dependent' and later 'hormone-responsive', cells in either state being unquestionably malignant and indefinitely transplantable in hormone-conditioned hosts; earlier still there are well-defined morphological changes, such as adenoma formation which, although 'benign' by all criteria, constitute a step on the road to malignancy. Thus, it does not seem possible to ascribe 'malignancy' in general to a single event which can convert a normal cell into a cancer cell. 'Premalignant' lesions are a characteristic feature of cancer in laboratory animals no less than in man. They are more commonly recognized where the tissue involved is readily accessible, as in the case of the breast or uterine cervix. The investigation of premalignancy in leukemia is feasible in the mouse, because the disease commonly starts as a localized focus in the thymus, characteristically in one lobe of the thymus (38). Histological changes in the thymus,
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Fig. 7. Cytotoxic tests with antisera detecting H-2, Thy-1, and Ly-2 alloantigens; comparative sensitivity of AKR thymocytes (from donors aged 2-6 mo old) and AKR thymic leukemia cells. Each line represents a titration with cells from a single donor.

Fig. 8. Estimation of the amount of H-2, Thy-1, and Ly-2 alloantigens by quantitative absorption analysis. Bold figures (under arrow) refer to the number of AKR thymocytes or thymic leukemia cells from individual donors required to reduce the cytotoxic activity of reference antisera to the same level (horizontal line) as congenic B6-H-2k, A-Thy-1.1, and B6-Ly-2.1 thymocytes from 2-mo-old donors. (See Table II and Materials and Methods).

spanning the period before frank malignancy, (i.e., before any clone of autonomously proliferating transplantable cells has appeared) have been described in detail (11, 38). Metcalf (11) summarizes these as cortical thinning accompanied by medullary enlargement with structures resembling lymphoid follicles. With
regard to biochemical changes, Kemp and Duquesnoy (39) have recently reported that adenylate cyclase levels are elevated in both leukemic as well as preleukemic (>6 mo) AKR thymus. From the present work, we now see that thymocytes also undergo characteristic antigenic changes in their surface phenotype during the preleukemic phase. By 6 mo of age, expression of several MuLV-related antigens (Glu, GCSA, gp70, and p30) on AKR thymocytes may be sharply increased. The pattern of alloantigen representation is also significantly altered in the preleukemic thymus. Whereas thymocytes from young adult mice characterized by high Thy-1/low H-2 expression, the reverse (low Thy-1/high H-2) is found on thymocytes from 6-mo-old AKR mice. Comparable age-related change in surface phenotypes has not been observed in tests with thymocytes from a number of strains having a low incidence of leukemia.

There are several possibilities to account for these changes in AKR mice: (a) Replacement of Thymus by Cells of a Different (e.g., Non-T) Lineage. This possibility can be excluded. Thymocytes from 2-mo and 6-mo-old

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Fig. 9. Quantitative differences expressed as an absorption index in the amount of H-2 antigens on thymocytes from AKR and B6-H-2 mice of various ages and on AKR thymic leukemia cells. Each symbol represents results obtained with cells from a single donor. Reference cells for calculating absorption index: B6-H-2 thymocytes from 2-mo-old donors.
Fig. 10. Quantitative differences expressed as an absorption index in the amount of Thy-1 antigen on thymocytes of AKR mice of various ages and on AKR thymic leukemia cells. Each symbol represents results obtained with cells from a single donor mouse. Reference cells for calculating absorption index: A-Thy-1.1 thymocytes from 2-mo-old donors. As the thymus of 6-mo-old A-Thy-1.1 mice is exceedingly small, it was not possible to obtain meaningful determinations of Thy-1.1 levels. For control purposes, B6 mice (Thy-1.2) were studied; thymocytes from 2 and 6-mo-old donors showed comparable levels of Thy-1.2 antigen.

AKR mice are of similar size and bouyant density, show comparable cortisone sensitivity, and in direct cytotoxic tests >90% are marked by Ly antigens characteristic of T cells.

(b) Replacement of Thymus by Leukemia Cells. This is excluded by the failure of thymocytes from 6-mo-old donors to produce leukemia after transplantation to young recipients.

(c) Preleukemic Changes in the Thymic Cell Population at High Risk for Leukemia Transformation. Current evidence favors this possibility. Surface markers and physical characteristics indicate that cells obtained from 2-mo and 6-mo-old AKR thymus belong to the same lineage and represent the predominant cell population, cortical lymphocytes. Immunofluorescence on sections of thymus has localized the increase of MuLV-related antigens in 6-mo-old AKR mice to the thymic cortex. The thymic cortex is also the predominant site of leukemia development in AKR mice. Thus, the cell population that is undergoing the age-dependent change in surface phenotype also appears to be the one that is at highest risk for leukemia transformation.

Lymphoid cells in the thymic cortex are known to be derived from precursor
cells that originate in the bone marrow (40). Under the influence of the thymic environment (presumably via a humoral factor produced by thymic stromal cells), the immigrant cells engage in intense mitotic activity and undergo the phenotypic changes (GIX⁻, TL⁻, Ly⁻, Thy-1⁻ → GIx⁺, TL⁺, Ly⁺, Thy-1⁺) that characterize thymic lymphocytes. With regard to the modified expression of MuLV-related antigens and alloantigens on preleukemic thymocytes, it may be asked whether this reflects age-dependent changes in bone marrow precursor cells or in cells of the thymic stroma. Experiments with thymic grafts or involving radiation chimeras reconstituted with bone marrow from 2-mo or 6-mo-old AKR donors provide ways to approach this question.

Previous studies have not found significantly higher MuLV titers in thymus of 6-mo-old AKR mice as compared to younger mice (9). This is surprising in view of the age-associated increase in MuLV-related antigens on AKR thymocytes. In collaboration with Doctors J. W. Hartley and W. P. Rowe of the National Institutes of Health, we have recently re-examined the issue of virus production by AKR thymus in relation to age. In accord with previous studies (9), the amount of mouse-tropic MuLV in 2-mo and 6-mo-old AKR thymus was not consistently different. Titers of xenotropic virus (as measured by fluorescent antigen induction in mink lung cells) were far higher in 6-mo-old AKR thymus.
and correlated with amplified expression of MuLV-related antigens.

In the study of the two naturally occurring oncogenic viruses of the mouse, mammary tumor viruses and MuLV, considerable advances have been made in our understanding of the factors that control susceptibility or resistance to these viruses and to the tumors they induce. Much has been learned about the genetic factors that determine virus production and dissemination, about immunological restraints on tumor development and growth, and about the hormonal influences that are involved in target cell susceptibility to viral transformation. Cancer development is also markedly age dependent, the incidence of mammary tumors and leukemia increasing with age. How this influence of age on cancer susceptibility is mediated remains unknown and is most probably part of the broader issue of age-related changes in the genetic control of differentiation. Further analysis of the defined changes in preleukemic AKR thymus may provide fresh insights to an understanding of this problem.

Summary

Thymocytes from preleukemic AKR mice aged 5-6 mo have an altered pattern of cell surface antigens. The expression of four MuLV-related antigens on the cell surface (Gp×, GCSA, gp70, p30) is markedly increased in comparison to 2-mo-old AKR mice and approximates the heightened levels of these antigens found on thymic leukemia cells. H-2 and Thy-1 alloantigens also show characteristic modifications in relation to age and leukemia development. In contrast to the high Thy-1/low H-2 levels on 2-mo-old AKR thymocytes, thymocytes from 6-mo-old mice and thymic leukemia cells frequently show a low Thy-1/high H-2 surface phenotype. As thymocytes from mouse strains with a low incidence of leukemia do not show these changes, they appear to represent a stage in the conversion of normal cells to leukemia cells.

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