ABSTRACT

AKR mice were inoculated with Gross murine leukemia virus at 3 days of age. Such treatment results in thymic lymphoma in all animals between 8 and 12 weeks of age. Normal AKR mice develop the disease after 28 weeks of age. In this study, virus-treated (AKR-V) and normal (AKR-N) mice were compared. Thymic weights, histology, and cell density as well as functional and mitogen assays of thymocytes and peripheral T-cells were studied. These animals were studied in the fifth week of life, a time of peak thymic size, and when the virus-inoculated mice were not yet leukemic but were a few weeks away from development of their disease.

The AKR-V mice when compared with the normal animals had decreased thymic weights and decreased total thymic cell density due to a loss of cortical cells. Their thymocyte response to the mitogen, phytohemagglutinin, and to alloantigen in mixed lymphocyte culture was increased significantly over that of cells from the AKR-N mouse thymus. Graft-versus-host reactivity of AKR-V thymocytes was also increased. The response to concanavalin A was significantly decreased in the AKR-V thymocytes. Treatment of the animals with cortisone resulted in a thymocyte population from both AKR-V and AKR-N mice with increased reactivity to phytohemagglutinin and concanavalin A as well as an increased responsiveness in the graft versus host reaction. T-cell responses from spleen and lymph node cells in all of the above tests were similar in the two groups, the single exception being that the AKR-V lymph node cells had a significantly higher background of thymidine uptake than did AKR-N cells. These observations suggest that loss in thymic weight of the virus-treated animals is due to a reduction of a subpopulation of cortical cells responsive to concanavalin A. The increased reactivity to phytohemagglutinin and alloantigen in the thymus is considered to be the result of a relative increase in reactive cells produced by the reduction of cortical thymocytes.

INTRODUCTION

The thymus is the target for the virus-induced neoplastic change that characteristically occurs in AKR mice, and it is also the organ responsible for the development of T-cells. Its study, just prior to lymphoma development, should determine if alterations in morphology and/or functional capacity precede neoplasia. This fact is important in our understanding of mechanisms of viral oncogenesis.

Several observations have been made in the thymus of AKR mice prior to lymphoma development. The peak weight of the organ is at 5 weeks of age followed by a steady decline in weight throughout the life of the animal (14). Old AKR mice develop a remarkable reduction in cortical cells with expansion of the medulla. The term "cortical inversion" has been used to describe the microscopic appearance, which is found in some animals. This describes a thymus in which the thymocyte content of the medulla is greater than that of the cortex. This cortical change has been shown to occur asymmetrically, i.e., one lobe is involved, when the other appears normal (20). The thymic changes are believed immediately to precede lymphoma development.

Murine leukemia virus expression changes with age in AKR mice. Xenotropic virus appears after 6 months of age (10). Expression of virus-related antigens has been shown to be markedly increased in 5- to 6-month-old AKR mouse thymi when compared to that found in 2-month-old animals (11). Also, new viruses with oncogenic properties are found in preleukemic thymus from older animals (8, 9). Studies of immunological function have indicated that cell-mediated immunity as measured by GVH reactions (17, 18) and T-cell-B-cell cooperation in the humoral response to sheep RBC (4, 16) are not impaired in young or old AKR mice. Age changes in mitogen responsiveness in AKR mice have been reported (17, 26).

In order to separate age-related structural and functional changes in the thymus from those specifically caused by oncogenic murine leukemia virus, we have used a model where preleukemic and normal animals are of the same age. When AKR mice are inoculated with GMuLV at 3 to 5 days of age, 100% of the animals die of leukemia between 8 and 12 weeks of life. Therefore, we have looked at thymic morphology, and at thymocyte and T-cell function in normal and virus-treated animals in the 5th week of life. We have found that GMuLV-mediated morphological changes are reflected by functional studies.

MATERIALS AND METHODS

Mice and Virus. The mice in this study were AKR, C57BL/6, and AKR × G57BL/6 F, hybrids from colonies maintained...
in the laboratory. They were caged in plastic boxes and given food pellets and water ad libitum. GMuLV was kindly supplied by Dr. Ludwik Gross and was maintained in continuous passage by inoculation of 3-day-old AKR mice i.p. with 0.1 ml of a cell-free filtrate of lymphomatous tissue from mice with virus-accelerated thymic lymphoma. The cell-free filtrate was prepared by (a) disruption of lymphoma cells, (b) centrifugation to remove cellular debris, and (c) filtration of the supernatant with a 0.45-μm-pore-size membrane filter. These filtrates contain GMuLV as well as the endogenous viruses of AKR mice. The cell-free filtrate was found to be free of contamination from other viruses (Dr. Michael Collins, Microbiological Associates, Bethesda, Md.). These viruses include pneumonia virus of mice, reovirus 3, Theiler’s encephalomyelitis virus, polyoma virus, K virus, ectromelia virus, minute virus of mice, Sendai virus, mouse adenovirus, mouse hepatitis virus, lymphochoriomeningitis virus, and lactic dehydrogenase virus.

In the present experiments, newborn AKR littermates were inoculated i.p. at 3 days of age with either 0.1 ml of a 0.9% NaCl solution (AKR-N) or 0.1 ml of a cell-free filtrate containing GMuLV (AKR-V). Mice were assayed for immunological reactivity in the fifth week of life. This single time period was chosen for 2 reasons, (a) the AKR-V thymus is truly preleukemic, i.e., free of lymphoma cells, as tested by cell transfer into syngeneic animals but certain to develop a lymphoma within 3 to 7 weeks if left in situ; (b) the normal AKR thymus at this age is at its peak size following an intense growth period and is just prior to age-related involution (14).

Cortisone Treatment. Mice were given 1.25 mg of cortisone acetate i.p. 72 hr prior to sacrifice for mitogen experiments and 48 hr prior to sacrifice for the GVH studies.

Histology. A group of 20 AKR-N and 20 AKR-V mice were sacrificed at 5 to 6 weeks of age. Their thymic weights were determined. The thymi were fixed in Bouin’s fluid and 4-μm-thick sections were prepared and stained with hematoxylin and eosin. Photomicrographs of representative sections were prepared by the UCLA Media Center.

Thymic Cellularity. The thymic cell density was determined by disrupting the entire thymus in a known volume of medium and preparing a suspension of individual cells, which were counted using a hemocytometer. To quantitate cell density as to thymic region, cells on hematoxylin-eosin-stained sections were counted at ×200 in 10 equally measured subcapsular (cortical) areas and 10 equally measured medullary areas from both AKR-V and AKR-N. A 1- x 1.5-cm rectangle fitted in the eyepiece of the microscope was used to make this assessment.

Mitogen Stimulation. Mice were sacrificed by cervical dislocation, and thymus, spleen, and lymph nodes were removed aseptically. In each experiment, tissues from 4 AKR-V and 4 AKR-N mice, 35 to 42 days old, were prepared separately. The cell suspensions were made by gently teasing the tissues apart in cold Dulbecco’s phosphate-buffered saline (Grand Island Biological Co., Santa Clara, Calif.). Tissue debris was allowed to settle for 5 min, and then cells were washed by centrifugation at 200 x g. The cell pellets were resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with penicillin, streptomycin, and 5% heat-inactivated fetal calf serum. The mitogens used were PHA (Difco Laboratories, Detroit, Mich.) and Con A (Calbiochem, San Diego, Calif.). The appropriate concentrations were prepared by diluting with medium. A microculture method was used consisting of plating 0.1 ml of either PHA (final concentration, 1:600) or Con A (final concentration, 2 μg/ml) in microtiter plates. Preliminary tests of PHA over a dilution range of 1:250 to 1:1000 and Con A over a concentration range of 1 to 10 μg/ml determined that 1:600 dilution and 2 μg/ml, respectively, resulted in maximal [3H]thymidine incorporation by thymus, spleen, and lymph node cells from AKR-N and AKR-V regardless of length of culture (2 to 96 hr) or the concentration of cells per well (0.3 x 10^6 to 2 x 10^7). Time optimum was determined to be 42 hr and cell optimum was 1.2 x 10^6/1000 and Con A over a concentration range of 1 to 7 μg/ml. Control cultures contained cells and medium alone. All tests were done in triplicate. Cultures were incubated for a total of 42 hr at 37° in humidified 5% CO_2-95% air including an 18-hr pulse with 2 μCi (0.05 ml) of [3H]thymidine (Schwarz-Mann, Orangeburg, N. Y.) per well. Cultures were harvested using a multiple automated sample harvester (MASH II) (Microbiological Associates, Los Angeles, Calif.) and prepared for scintillation counting. Data from 5 separate experiments were averaged, and S.E. were computed.

GVH Reactions. Varying numbers of thymus, spleen, and lymph node cells in 0.1-ml volumes from AKR-V and AKR-N mice were injected i.p. into AKR x C57BL/6 F mice 1 to 3 days old. The spleen index, computed according to the method of Simonsen (21), was obtained by killing injected mice and noninjected littermates 9 days after cell inoculation. An index of >1.3 was considered to be positive. Data were plotted as the spleen index versus the cell dose. Since GVH reactivity is a measurement of splenomegaly, lymphoma cells, if present in the AKR-V thymus, could, by replicating in the host spleen, give a spurious positive reaction. To test this possibility, AKR lymphoma cells were inoculated into 1- to 3-day-old F hybrids, and spleen indices were determined in the same manner as for the nonmalignant cells.

MLC. The same microculture methods were used as for mitogen cultures. Appropriate numbers of cells were pipetted into microwells in 0.1-ml volumes. Corresponding numbers of irradiated (1500-rad) stimulator cells were also added to wells in 0.1-ml volumes. Maximal stimulation for both AKR-N and AKR-V cells occurred when 0.6 x 10^6 cells were cultured with an equal number of stimulator cells. Stimulator cells were either C57BL/6 spleen cells (allogeneic) or AKR-N or AKR-V spleen cells (syngeneic). Cultures were incubated at 37° in humidified 5% CO_2-95% air for 42, 66, and 96 hr including an 18-hr pulse label with 2 μCi (0.05 ml) of [3H]thymidine. Maximal responses occurred at 66 hr. Cultures were harvested and processed as for mitogen cultures.

RESULTS

Morphological and Cellular Studies of AKR-V and AKR-N Thymus. The AKR-V thymus was found to be smaller than the AKR-N thymus [66 ± 2.9 (S.E.) mg versus 99 ± 4 mg]. This was accompanied by a lower total cell count for the AKR-V (1.25 x 10^7 ± 0.17) than for AKR-N thymus (2.30 x...
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10^4 ± 0.22). Both weight and cell count showed statistically significant differences using the Student's t test (p < 0.005). Examination of fixed tissue sections of AKR-V and AKR-N thymus demonstrated a moderate diffuse depletion of cortical cells in the virus-treated mice (Fig. 1). There were increased numbers of mitoses seen in the subcapsular area of the AKR-V thymus. Actual counts of 2000 thymic cortical cells on the slides revealed 1 and 0.3 mitosis per 100 cells for AKR-V and AKR-N thymus. The medullary area of the AKR-V thymus was greater than that found in the normal animals.

To document the observation of cortical cell thinning, quantitation of the cortical cells using actual counts of measured areas were carried out (10 areas of cortex and 10 areas of medulla from 3 separate AKR-N and AKR-V thymi). The AKR-N cortical cell number was 48.6 ± 7.0 (S.D.). The AKR-V cortical cell number was 29.7 ± 7 (S.D.). There was a highly significant difference in these cell numbers using the Student's t test (p < 0.001). The cell count of the AKR-N medulla was 19.9 ± 3.7 (S.D.), and the cell count of the AKR-V medulla was 17.7 ± 3.8.

It is important in studies such as these to ascertain whether lymphoma cells are actually present in the thymus of the AKR-V mice. To do this 10^4 AKR-V thymocytes were inoculated into newborn AKR mice. Newborn mice given injections of as few as 10 lymphoma cells will develop generalized lymphoma within 3 weeks. However, the mice given injections of AKR-V thymocytes developed lymphoma after 8 weeks of age. This is a time when a leukemogenic virus, if present in the cell inoculum, would be expected to induce the disease. To confirm this possibility, newborn AKR × C57BL/6 F1 hybrids were inoculated with 10^7 and 10^8 AKR-V thymocytes and compared with noninjected littermate controls. The 6 mice from 2 litters receiving AKR-V thymocytes developed lymphoma at 33 to 155 days post inoculation. None of the 5 noninjected controls developed lymphoma by 180 days when the experiment was terminated. The lymphomas were tested by cell transfer into AKR and F1 mice. They produced lymphomas in all of the hybrids within 3 weeks. The cell-inoculated AKR mice did not develop lymphoma after 8 weeks of observation. This confirms the fact that the lymphomas developing after inoculation of the AKR-V thymus cell suspension were virus induced in the hybrid host cells and not the result of the transfer of a small number of malignant AKR cells. Thus, we considered that the AKR-V thymus was truly preleukemic.

Mitogen Studies of AKR-N and AKR-V Thymus with and without Cortisone Pretreatment. The responsiveness of thymocytes from AKR-V or AKR-N mice at various cell concentrations to PHA is shown in Chart 1. The peak response for both occurred at 1.2 x 10^6 cells/well. At that cell concentration, AKR-V thymocytes had 2-fold greater proliferative response to PHA than did AKR-N thymocytes (p < 0.05; Student's t test).

Responsiveness to Con A of AKR-N and AKR-V thymocytes is illustrated in Chart 2. The maximum response to Con A also occurred at 1.2 x 10^6 cells/well. However, the AKR-N thymocytes response to this mitogen was significantly greater than that of AKR-V thymocytes (p < 0.05; Student's t test).

The effect of treatment with cortisone on the AKR-N and AKR-V thymus was evaluated. Data in Table 1 demonstrate that the cortisone affected the AKR thymus in a similar way to that described in other strains of mice, i.e., enhanced responsiveness to both PHA and Con A (22). When comparing AKR-N and AKR-V thymus with and without cortisone treatment, a similar change for each mitogen was found, i.e., a greaterPHA response and a lesser Con A response in the AKR-V thymus. Both AKR-N and AKR-V thymus from cortisone-treated mice showed reduction in thymic weight and cell density, and extreme depletion of cortical cells when tissue sections were examined. There were no differences in morphology, weight, or cell counts in AKR-V or AKR-N thymus after cortisone treatment.

Response of Peripheral Lymphoid Tissue to Mitogens. These responses are illustrated in Table 2. A higher background (control) lymph node reactivity was found in the AKR-V mice. This was also observed in the syngeneic MLC lymph node preparation (Table 3). The control count was equally high in both AKR-N and AKR-V spleens. No significant differences in mitogen responses were demonstrated.

GVH and MLC Responses. The GVH potential of thymocytes from AKR-V and AKR-N mice was investigated, and the results are illustrated in Chart 3. Clearly, the ability of thymocytes from AKR-V mice to induce splenomegaly in F1
Table 1
Mitogen responsiveness in AKR-N and AKR-V thymus with and without cortisone pretreatment
All data represent responses of 1.2 × 10⁶ cells/well.

<table>
<thead>
<tr>
<th></th>
<th>AKR-N</th>
<th>AKR-V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PHA</td>
</tr>
<tr>
<td>cpm</td>
<td>803 ± 143</td>
<td>2,666 ± 361</td>
</tr>
<tr>
<td>After cortisone</td>
<td>554 ± 73</td>
<td>34,256 ± 6,630</td>
</tr>
</tbody>
</table>

* Normal 5-week-old mice.
* Five-week-old mice neonatally inoculated with GMuLV.
* Mean ± S.E.
* 72 hr after 1.25 mg cortisone acetate i.p.

Table 2
Mitogen responses of spleen and lymph node of AKR-N and AKR-V mice
All data represent responses at 1.2 × 10⁶ cells/well.

<table>
<thead>
<tr>
<th></th>
<th>AKR-N</th>
<th>AKR-V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PHA</td>
</tr>
<tr>
<td>Spleen</td>
<td>14,285 ± 1,318</td>
<td>64,101 ± 8,489</td>
</tr>
<tr>
<td>Lymph node</td>
<td>2,615 ± 421</td>
<td>74,515 ± 9,301</td>
</tr>
</tbody>
</table>

* Normal 5-week-old mice.
* Five-week-old mice neonatally inoculated with GMuLV.
* Mean ± S.E.

Table 3
MLC responsiveness of thymus, spleen and lymph node of AKR-V and AKR-N mice
All data represent responses at 1.2 × 10⁶ cells/well.

<table>
<thead>
<tr>
<th></th>
<th>AKR-N</th>
<th>AKR-V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Syngeneic</td>
<td>Allogeneic</td>
</tr>
<tr>
<td>Thymus</td>
<td>414 ± 49</td>
<td>1,337 ± 72</td>
</tr>
<tr>
<td>Spleen</td>
<td>13,329 ± 1,497</td>
<td>39,420 ± 4,841</td>
</tr>
<tr>
<td>Lymph node</td>
<td>6,551 ± 485</td>
<td>39,981 ± 3,358</td>
</tr>
</tbody>
</table>

* Normal 5-week-old mice.
* Five-week-old mice neonatally inoculated with GMuLV.
* Mean ± S.E.
hybrid recipients was significantly greater at higher cell
doses. The increased reactivity was not due to proliferation
of lymphoma cells as discussed above. In addition, 5 x 10^6,
1 x 10^6, or 1 x 10^5 lymphoma cells from AKR mice with
GMuLV-accelerated lymphoma were inoculated into F1
mice. Of 29 animals inoculated, 28 had spleen indices of
less than 1.3. One mouse given 10^6 lymphoma cells had a
spleen index of 1.3. These findings indicate that lymphoma
cells, and/or the GMuLV they express, could not produce
splenomegaly.

Cortisone treatment 48 hr before donor sacrifice was
found to enrich the AKR-N and AKR-V thymus for GVH-
reactive cells. When 5 x 10^6 cells from cortisone-treated
animals were used, the mean spleen index from 4 AKR-N
thymus-inoculated litters was 2.4 ± 0.5 (S.E.) and that from
4 AKR-V litters was 1.48 ± 0.09 (S.E.). GVH reactions were
not produced by 5 x 10^6 thymocytes in AKR-N animals, and
they resulted in a minimal reaction in AKR-V animals
not given steroids (Chart 3).

The MLC is considered to be the in vitro correlate of GVH,
and the results corroborate the latter. As shown in Table 3,
the in vitro reactivity of AKR-V thymocytes to alloantigen
was significantly greater than that of AKR-N thymocytes.
Studies of spleen and lymph node cells with GVH as well as
MLC showed no differences in reactivity between AKR-N
and AKR-V. MLC studies of the thymus after cortisone
treatment were not done.

DISCUSSION

This work has shown that the thymus of mice inoculated
with GMuLV at 3 to 5 days of age, when studied in the 5th
week of life, has a significant reduction of weight and
cellularity when compared to the age-matched normal thymus.
This is the result of a diffuse loss of cortical thymocytes
cortical thinning).

The mitogen studies reflect this population change of the
AKR-V thymus. The PHA-reactive cell is concentrated in the
thymus medulla (1); therefore, a process causing cortical
thinning will result in increased numbers of these cells in
pooled thymocyte populations and an augmented PHA
response. Cells with Con A reactivity are known to be
present in both cortex and medulla of the thymus (23). The
diminished Con A response found in the AKR-V thymocytes
suggests that the cells lost from the thymic cortex are a
population reactive to Con A. The findings with cortisone
treatment strengthen this conclusion. The mitogen data
from AKR-N and AKR-V thymus, when compared to those
found with AKR-N and AKR-V cortisone-treated thymus,
show that GMuLV treatment produces a different effect
than does cortisone, i.e., a specific loss of cortical cells
with Con A responsiveness produced by the virus treatment
and a larger loss of mitogen-unresponsive cortical cells
produced by the steroid. These steroid-sensitive cells were
found in both AKR-N and AKR-V thymus.

GVH and its in vitro correlate the MLC reflect also the loss
of thymic cortical cells in the AKR-V mice, producing a
subpopulation of thymocytes more reactive in these func-
tional tests. The loss of cortisone-sensitive cells from the
thymus increased GVH responsiveness of cells from normal
and virus-treated mice.

Spleen and lymph node cells did not show a remarkable
change in response to mitogen or alloantigen in the virus-
treated mice. The spleen background was high in both
groups. This finding has been reported previously for AKR
spleen cells (25). The spontaneous thymidine uptake for
AKR-V lymph node cells was higher than that of the cells
from normal mice. Thus, virus treatment of AKR results in a
lymph node cell population with an increased in vitro
proliferative capacity.

Previous morphological studies of the thymus of AKR
mice prior to spontaneous lymphoma development (15, 18,
20) and of other strains inoculated with radiation leukemia-
virus (6) and Moloney murine leukemia virus (3) have
demonstrated a preleukemic cortical thinning, resulting in
a loss of thymic weight. However, the lesion described in
preleukemic old AKR mice demonstrated a more marked
loss of cortical thymocytes than was seen in the AKR-V
mice reported in our study. This could reflect a combination
of virus- and age-related changes in the old preleukemic
animal. The findings with our functional studies must be
evaluated in the context of published reports of T-cell and
thymocyte responses in AKR mice. A progressive loss of
responsiveness to PHA and Con A in AKR spleen and lymph
node cells has been shown to occur with age (5, 26). In this
respect, AKR mice behave like animals of other strains in
which an age-related decline in mitogen reactivity has been
described (13). Cell-mediated immune responses, GVH
reactivity of spleen cells in particular, have been found to
be unchanged throughout life in AKR mice (7, 12). Two
studies measuring mitogen responsiveness of young and
old "preleukemic" AKR thymocytes have demonstrated
increases in PHA responsiveness and decreases in respons-
siveness to Con A in the thymus cells of the old mice (17,
26). These findings are similar to ours in the young animals.
This is evidence that the changes in mitogen reactivity
observed in the thymus are specifically preleukemic and
virus-induced and not age-related.
Our studies not only indicate a GMuLV-mediated loss of a subpopulation of thymic cortical cells but also demonstrate normally functioning medullary thymocytes and peripheral T-cells in the virus-treated mice. These findings suggest that the virus is affecting a population of cortical cells that does not give rise to medullary thymocytes. There is controversy regarding the pathways of thymocyte maturation (19, 24). Shortman has presented evidence for a cortical cell population with a maturation pathway separate from that of medullary cells. This cortical population is made up of at least 2 subpopulations, and their function is not known (19). The subcapsular area of the thymus cortex is where larger cells with more frequent mitoses are seen in the AKR-V mice and where the initial leukemic transformation most probably occurs (2). We propose that this area may represent a part of the thymus giving rise to a virus-sensitive thymocyte subpopulation responsive to Con A and separate from the high-α, cortisone-sensitive, mitogen- and alloantigen-unresponsive major cortical cell population.

REFERENCES
Gross Murine Leukemia Virus-induced Alterations in the Thymus of Preleukemic AKR Mice

Terry R. Beardsley and Esther F. Hays