Mechanisms of Thymic Lymphomagenesis by the Retrovirus SL3-3

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Abstract

These studies report changes occurring in the thymus of AKR and NFS/N mice after infection with the lymphomagenic retrovirus SL3-3. In virus-infected AKR fetal thymus, the programmed cell death caused by treatment with antibody to CD3 was remarkably diminished. A method of establishing thymic stromal cultures from mice of 1 to 3 wk of age is described. Using this method, it was found that SL3-3 virus infection by neonatal inoculation allowed establishment of thymic stromal cultures from organs removed from AKR mice of 30 to 50 days of age and from lymphomas, whereas thymic stromal cultures could not be established from control mice after 30 days of age. Using NFS/N mice which have no endogenous virus, it was shown that infection of thymic stroma precedes infection of thymocytes and that thymocytes are permissive for infection with SL3-3 virus but not for the nononcogenic retrovirus, Akv, yet Akv virus replicates efficiently in thymic stroma.

SL3-3 virus integrates randomly in each lymphoma induced by this virus. The lymphomas are clonal or oligoclonal. Plm-1 and c-myc genes commonly rearranged in other virus-induced thymic lymphomas showed rearrangement in only a few lymphomas.

A theory is proposed, based on the work presented here and in recent studies, which states that SL3-3 virus infection of thymic stroma allows infection of thymocyte progenitors entering from the bone marrow. These cells are then altered so that their maturation is delayed and their intrathymic survival is prolonged. This permits virus integration and reintegration that results in the genetic changes which transform the cell.

Introduction

The mechanism of induction of lymphoma by the non-acute transforming retroviruses has not yet been clearly defined. The most complete data on this subject comes from studies of an avian leukemia virus-induced B-cell lymphoma. They have provided evidence of retroviral insertion mutagenesis (1) and shown that tumor progression can occur as a result of stage-specific oncogene activations (2). The spontaneous thymic lymphoma of AKR mice is also caused by non-acute transforming retroviruses; however, the mechanism of virus-induced transformation is not known. In order to study this phenomenon, our laboratory has used the highly oncogenic retrovirus SL3-3. This virus was isolated from a cell line established in this laboratory from a spontaneous AKR lymphoma (3). This virus, which has been molecularly cloned, when inoculated by the i.p. or i.t. route into newborn AKR mice, induces a 100% incidence of thymic (T-cell) lymphoma in animals 60 to 90 days of age. The nucleotide sequences of the structural genes of SL3-3 virus are essentially similar to those of the nononcogenic endogenous virus of AKR mice, Akv (4). The differences lie in the noncoding LTR region, and the LTR of SL3-3 has been shown to be responsible for its properties of tropism for thymocytes and oncogenicity (5, 6).

The stromal cells of the AKR thymus are highly permissive for SL3-3 virus infection (7); thus, they provide a source of virus for infection of immature thymocytes, i.e., cells which enter the thymus in an undifferentiated state from the bone marrow.

Abnormal thymocyte/stromal interaction in virus-infected mice has been demonstrated using fetal thymus organ cultures (8). In these experiments 14-day fetal thymi were treated with deoxyguanosine to remove lymphocytes and then incubated with double negative (absence of CD4 and CD8 markers) thymocytes from virus-infected and normal 50-day-old animals. Maturation was shown with the thymocytes of normal AKR mice by the appearance of CD4 and CD8 markers. Double negative thymocytes from virus-infected thymi did not mature in these fetal thymus organ cultures. In studies of the normal-appearing thymus during the 60-day period after SL3-3 virus infection and before lymphoma develops, variations in thymocyte subpopulations were seen. The first changes occurred at 37 days of age, and by 50 days of age, nearly every thymus was abnormal (9). A common feature was a reduction in size of the large CD4+ CD8+ (double positive) thymocyte population. Lymphoma cells were found in thymi from animals of 49 to 60 days of age. Interestingly, two stages of lymphomagenesis were defined, i.e., TD cells, which produced donor-type lymphoma after a long latency (median, 54 days) after i.t., but not s.c. inoculation, and TI cells, which produced donor-type lymphoma within 3 to 4 wk after inoculation by both routes (9). In the development of thymic lymphoma, the TD cells precede the TI cells which have the same transplantation characteristics as do tumor cells. This process of tumor progression was shown to develop independently in individual thymic lobes (9). Isolation of double negative cells from virus-infected thymi separated TD from TI cells (8). Clonality or oligoclonality of cells at TD, TI, and tumor stages was shown by studying genomic DNA for rearrangements of the J-β2 T-cell receptor gene (Ref. 9; Footnote 4).

This paper describes studies of mouse thymocytes and stromal cells from thymi of AKR and NFS/N (a strain with no endogenous virus yet susceptible to lymphomagenesis by SL3-3 virus). Changes produced in them by SL3-3 retrovirus infection are shown. Preliminary studies of genomic DNA from AKR and NFS/N lymphomas for virus integrations and oncogene rearrangements are also presented.

Materials and Methods

Mice. The AKR/J and NFS/N mice used in these studies are from inbred colonies maintained in the laboratory. The AKR × DBA/2 F1 (hereafter called AKD2F1) hybrids were also bred in the laboratory.

Fetal Thymus Organ Cultures. Fourteen- to 15-day-gestation fetal thymi were removed aseptically using a dissecting microscope. Fetal age was determined from the time of appearance of the vaginal plug. Six to 8 thymi were placed on nuclepore filters supported by Gelfoam rafts in 35-mm culture dishes with growth medium and 10% fetal bovine serum. SL3-3 virus infection was carried out just prior to culture by puncturing the capsule with a fine gauge needle in a small amount of medium containing 7 × 10^6 plaque-forming units/ml of virus. Poly-
**SL3-3 VIRUS LYMPHOMAGENESIS**

Brene was added to these cultures. They were incubated at 37°C for 2 h, washed, and placed on the filters for culture. Control thymi were treated in the same way in the absence of virus. After the culture period of 8 days, certain thymi were removed, and the thymocytes were teased from them and assayed for surface gp70, CD4, and CD8 using monoclonal antibodies and fluorescence flow cytometry. Additionally, thymi were left in culture, and equivalent amounts of anti-CD3 and anti-Thy 1.1 were added to experimental and control cultures which were incubated an additional 18 h. At the end of this period thymi were removed for formalin fixation and the preparation of 1-μm hematoxylin- and eosin-stained sections. Cell suspensions were made from some thymi which were counted and analyzed for CD4 and CD8.

**Culture of Thymic Stromal Cells.** The thymus was removed and pressed through a stainless steel screen. The material on top of the screen consisted of thymic capsule, attached stroma, and thymocytes. This material was washed vigorously to remove as many thymocytes as possible. Then it was treated with collagenase-dispase (100 μg/ml) in phosphate-buffered saline with DNase I (50 μg/ml) at 37°C for 30 min to prepare a cell suspension. In some experiments the cells from a single thymus were placed in a 35-mm culture dish and, in the experiments where quantitation was necessary, the cells were divided among 8 wells in a 24-well plate. All culture plates were previously coated with a matrix consisting of bovine serum albumin (10 μg/ml) and collagen (vitrogen; 30 μg/ml). The growth medium consisted of low-glucose Dulbecco’s modified Eagle’s medium with L-glutamine, and penicillin-streptomycin with added hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), selenium (5 μg/ml), transferrin (5 μg/ml), and 10% fetal bovine serum. The medium was changed at 3-day intervals by replacing one-half of the spent medium with fresh medium.

**Assessment of gp70 on Thymic Stromal Cultures.** The culture medium was removed, and the attached cells were fixed and stained with a double antibody fluorescence technique. The presence of surface gp70 was determined using an inverted fluorescence microscope.

**Recombinant Retroviruses.** Three recombinant retroviruses were used and compared with molecularly cloned isolates of intact SL3-3 and Akv viruses. pRECAS-8 was composed of the entire env gene and most of the pol gene of Akv virus. Part of the pol, all of the gag gene, and the LTRs were from SL3-3 virus. pRECAS-20 was the reciprocal of pRECAS-8; thus, it contained the Akv virus LTR (10), and RECAS-LTR contained the SL3-3 virus LTR and the Akv coding sequences (5).

**Southern Blot Analysis.** Genomic DNA from tissues was isolated by lysing the cells in 50 mM Tris (pH 9.0), 10 mM EDTA, 1% SDS, and 100 μg/ml of proteinase K for 2 h at 50°C and extracted with phenol/chloroform. Fifteen μg of genomic DNA were digested with the appropriate restriction enzyme, separated on agarose cells, transferred with the spent medium with fresh medium.

**Results**

**Effect of Antibody to CD3 on Survival of Normal and Virus-infected Thymocytes.** These studies were initiated following the report of Smith et al. (14) that antibody to CD3, a molecule which is part of the T-cell receptor complex, caused programmed cell death of fetal thymocytes in organ culture. Cell death is measured by the appearance of apoptosis in thin microscopic sections, reduction of thymocyte numbers, especially of the double positive subset, and degradation of DNA. This response of anti-CD3 probably represents the way thymocytes respond to self antigens, i.e., the process of negative selection. Experiments were carried out to look at the effect of SL3-3 virus infection on the interaction of anti-CD3 with fetal thymocytes. Fetal thymocytes from normal AKR mice, after 7 to 8 days in culture, are negative for expression of the viral envelope protein, gp70. When these organs were treated in vitro with SL3-3 virus and cultured for the same period of time, 40 to 60% of the cells become gp70 positive, indicating infection with the virus. After 7 to 8 days in culture, the cells from the normal thymi were 74% CD3 positive, and 80% had both CD4 and CD8 markers on their surface. When these normal thymi were treated with anti-CD3, extensive apoptosis of thymocytes but not of stromal cells was seen. Thymi cultured with anti-Thy 1.1 as a control antibody showed no cell death. The double positive thymocyte population from the anti-CD3-treated cultures was less than half that of cultures treated with anti-Thy 1.1 (23 versus 75%). When virus-infected cultures were treated with anti-CD3, small foci of apoptosis interspersed with larger areas of intact thymocytes were seen. The double positive population was reduced from 22%, prior to incubation with anti-CD3, to 8% after the treatment. Apoptosis again was not present when anti-Thy 1.1 was used. There was a greater decrease in the number of thymocytes after anti-CD3 treatment of the uninfected thymi. DNA from virus-infected thymi but not from virus-infected thymi showed degradation on gels. The observation of less programmed cell death after anti-CD3 treatment in the virus-infected fetal thymus was repeated in three separate experiments. Thus, these experiments show, in the fetal thymus system, that SL3-3 virus can effect change in thymocytes; i.e., the programmed cell death occurring in response to anti-CD3 is diminished, and the double positive population is reduced.

The lymphomagenic properties of the SL3-3 virus were also demonstrated in this system. Fifteen-day-gestation AKR fetal thymus, after virus infection and 8-day culture, was grafted under the kidney capsule of AKD2F mice. Lymphoma of the donor type, i.e., Thy 1.2 negative and Thy 1.1 positive, developed 2 mo later at the site of the graft.

**Effect of SL3-3 Virus on the In Vitro Growth of Thymic Stromal Cells.** The development of lymphoma in the AKR mouse thymus depends on an interaction between thymic lymphocytes and thymic stromal cells in the presence of oncogenic virus. Using a method of thymic stromal culture which consistently produced cultures which survive for several months, we were able to observe the effect of virus infection on stromal cell growth. These stromal cells showed the pavement-like morphology of epithelial cells. They were major histocompatibility complex Class II positive, Class I negative, and keratin negative. After primary explant, the stromal cells formed islands or colonies which became confluent after 12 days of culture (Fig. 1). Stromal cultures from AKR mice of different ages neonatally infected with SL3-3 virus were studied. Stromal cells from both virus-infected and normal mice under 30 days of age formed 20 to 30 colonies after 10 days of culture. After 30 days of age, thymi from normal mice produced 0 to 2 stromal colonies, whereas stromal cultures from 40- to 50-day-old SL3-3 virus-infected mice, like those from the young mice, developed 20 to 30 colonies. Many stromal colonies were also found in cultures prepared from thymic lymphomas. Interestingly, the cultures from neonatal thymic stroma of AKR mice were gp70 negative. The cultures from both normal and SL3-3 virus-infected mice became gp70 positive within the first 2 wk of life. Thus, we showed that oncogenic virus infection, but not endogenous virus infection, enhanced the in vitro growth of stromal cells from thymi of mice over 30 days of age and from lymphomas.

Spread of SL3-3 Virus in Thymocytes and Stroma of Mice of the Endogenous Virus-free NFS/N Strain. The NFS/N strain was used in these experiments because it had no endogenous virus background yet was highly susceptible to lymphoma.
the two viruses were $2 \times 10^5$ plaque-forming units per ml. The results, which are summarized in Table 1, were as follows. At 7 and 9 days, some stromal cells from SL3-3 virus-treated mice were positive. All cells were positive at 12, 14, and 21 days of age. Stromal cells from the Akv-treated mice were positive in all 8 wells at each age period. The thymocytes from animals treated with both viruses were gp70 negative at 7 and 8 days of age; they became increasingly positive (25 to 60%) in SL3-3-treated mice between 12 and 21 days of age. In contrast, thymocytes from Akv virus-treated mice remained negative at 12 and 14 days of age and were 5% positive at 21 days of age. Stromal cell cultures and thymocytes from normal NFS/N mice did not express gp70 at any age. This is a clear demonstration

Table 2 Oncogenicity of recombinant retroviruses in AKR mice

<table>
<thead>
<tr>
<th>Virus designation</th>
<th>No. of mice with thymic lymphoma/no. of mice inoculated</th>
<th>Incidence (%)</th>
<th>Mean latent period (days)</th>
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<tr>
<td>pRECAS-8</td>
<td>7/7</td>
<td>100</td>
<td>118</td>
</tr>
<tr>
<td>pRECAS-20</td>
<td>0/15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RECAS-LTR</td>
<td>8/13</td>
<td>61</td>
<td>130</td>
</tr>
<tr>
<td>SL3-3</td>
<td>11/11</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>Akv</td>
<td>0/15</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

SL3-3 or Akv virus (10⁸ plaque-forming units) was inoculated i.p. into mice less than 24 h of age. There were 4 mice studied at each time period.

genesis with SL3-3 virus. Eighty % of mice inoculated i.p. within 24 h of birth develop thymic lymphoma between 60 and 90 days of age. In this study, mice were inoculated with virus at 24 h of age; they were killed at 7, 9, 12, 14, and 21 days of age; and the thymus was removed for study. Thymocytes were harvested from the same organs used for stromal culture and assayed for gp70 using fluorescence flow cytometry.

Table 1 gp70 expression on thymic stroma and thymocytes of virus-treated and normal NFS/N mice

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>% of gp70+ stromal cells</th>
<th>% of gp70+ thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SL3-3*</td>
<td>Akv</td>
</tr>
<tr>
<td>7</td>
<td>2-3</td>
<td>100</td>
</tr>
<tr>
<td>9</td>
<td>50-100</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
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<td>14</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* SL3-3 or Akv virus (10⁸ plaque-forming units) was inoculated i.p. into mice less than 24 h of age. There were 4 mice studied at each time period.
Normal Maturation of Thymocytes

Thymic cortex

Thymic medulla

PBL

DN → Im CD3

CD8+ → CD4+

CD4+

CD8+

CD4+

CD8+

Pre T

Bone Marrow

Fig. 4. Normal maturation of thymocytes. Cells without T-cell receptor gene rearrangement enter the thymus from the bone marrow (pro T, pre T). They mature to cells expressing CD3 but negative for CD4 and CD8 (double negative, DN). The CD8 molecule is then expressed. The next step in the maturation is the expression of CD4 and CD8 on CD3-positive thymocytes. Most of these cells die within the thymus. A small percentage of them express either CD4 or CD8 and migrate to the thymic medulla from where they move to the peripheral blood and become mature CD4+ and/or CD8+ peripheral blood lymphocytes (PBL). The size of the circle represents the size of the thymocyte cell pool with the surface phenotype indicated.

Abnormal Maturation of SL3-3-infected Thymocytes

Thymic cortex

Thymic medulla

PBL

DN → Im CD3

CD8+ → CD4+

CD4+

CD8+

CD4+

CD8+

Pre T

Bone Marrow

Fig. 5. Abnormal maturation of SL3-3-infected thymocytes. SL3-3 virus alters the maturation progression with resulting change in cell pool size; i.e., the immature DN and CD8+ cells are increased, and a decrease in the double positive cells is seen. Some cells undergo normal maturation. Virus infection (△) is indicated by expression of surface gp70.

of stromal cell permissiveness for infection with both viruses, thymocyte tropism of SL3-3 virus, and stromal cell infection with retrovirus preceding that of thymocytes.

Genomic Virus Integration as a Mechanism for SL3-3 Virus-induced Lymphomagenesis. The preceding sections have described alterations in the thymus as the result of oncogenic retrovirus infection. In this section we describe studies designed to evaluate genetic changes associated with virus integration in the lymphoma cell DNA. Their purpose is to define a number of gene alterations which may explain why virus infection consistently causes malignant transformation of thymocytes. Studies have shown that the LTR region of the SL3-3 viral genome differed from the Akv viral LTR and that the differences were in the enhancer region of the LTR (5). Recombinant retroviruses (provided by Dr. Jack Lenz) were tested in our mouse strains to confirm this observation. As shown in Table 2, when the recombinant virus contained the Akv LTR and the SL3-3 viral genome, it was not oncogenic, whereas the SL3-3 LTR conferred oncogenic properties to the Akv genome.

In subsequent experiments, genomic DNA was prepared from 30 SL3-3 virus-induced thymic lymphomas of AKR and NFS/N mice. Southern blot analysis of thymoma DNA, using a probe specific for the env gene of Akv virus (15), showed newly acquired SL3-3 viral DNA in each tumor (Fig. 2). Normal NFS/N mice had no viral DNA in their thymus, while blot from normal AKR mice showed the four germline bands of Akv DNA. When the DNA is digested with EcoRI, which does not cleave the viral genome, bands larger than 8.2 kilobases indicate the presence of a full-length viral genome. With the probe used, the tumor DNA showed one or a few new viral integrations per tumor. The bands were all larger than 8.2 kilobases (Fig. 2). These findings indicate the presence of full-length viral DNA in the tumors and that they are monoclonal or oligoclonal. These studies also showed that no one region was a frequent site of virus integration; however, there was some evidence that a particular region may be a target in more than one tumor. Thymic lymphomas induced by the Moloney leukemia virus in mice have been found to have frequent rearrangements of the pim-1 and c-myc genes due to insertional mutagenesis by the viral DNA (12, 16). Therefore, our genomic DNA preparations were screened with pim-1 and myc gene probes to look for rearrangements. Pim-1 was rearranged in 0 of 30 AKR tumors (data not shown) and in 1 of 30 NFS/N tumors (Fig. 3). The c-myc gene was rearranged in 5 of 24 AKR tumors and in 7 of 24 NFS/N tumors (data not shown). The same tumor had rearranged pim-1 DNA in both EcoRI and EcoRV digests (Fig. 3), and rearranged c-myc DNA was found in the same tumors with EcoRI and KpnI digests. Thus, it appears that genes other than pim-1 and c-myc are involved in the induction of lymphoma by SL3-3 virus.

Discussion

It is apparent from the data presented here that the mechanisms of lymphomagenesis that regularly occurs after infection with SL3-3 virus are complex and that the development of this disease occurs in stages. The first stage is an alteration of the thymic stroma after virus infection as demonstrated by our stromal culture data. Data to support our findings are found in the observation of proliferation of a thymic stromal population in spontaneous lymphomas of AKR mice defined by a monoclonal antibody (17). Stromal infection, which alters thymocyte-stromal interactions, we believe results in the next stage, which is the development of thymocytes with the capability for prolonged i.t. survival, as shown by the appearance of thymus-dependent lymphoma cells, which produce lymphomas with a median latency of 54 days after i.t. inoculation (9), and the prolonged latency of donor-type lymphoma development after the engraftment of virus-infected fetal thymus remnants. Normal immature thymocytes do not self renew and remain within the thymus for only 14 to 21 days after emigration from the bone marrow and before export to the peripheral lymphoid tissues (18). This abnormal i.t. sojourn of virus-infected cells we believe results in an increase in the double negative and immature CD8 thymocytes and a consistent decrease of the large double positive pool of thymocytes. Immature self-renewing cells become targets for virus integration and reintegration,
thus permitting the genetic changes necessary for malignant transformation to take place.

Figs. 4 and 5 illustrate in simplified diagrams the changes in normal thymocyte development which occur in the oncogenic virus-infected thymus. It is the goal of future experiments to further define the i.t. prelymphoma cells and to demonstrate meaningful genetic changes associated with viral integration in the lymphoma cells.

References

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