Erythroid Leukemia Induced by Friend Lymphatic Leukemia Virus in T-Cell-depleted Mice

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ABSTRACT

BALB/c mice depleted of T-cells by thymectomy at 3 to 5 days of age and by treatment with antithymocyte serum were inoculated with the lymphatic leukemia virus derived from Friend virus. After a long latent period, these animals developed erythroid leukemia. In contrast, intact control mice inoculated with Friend virus-associated lymphatic leukemia virus developed typical thymic (T-cell) lymphomas. Cell-free virus prepared from leukemic T-cell-depleted animals induced lymphoid, myeloid, and erythroid leukemias in intact mice. The erythroid leukemia-inducing virus differed from the spleen focus-forming component of Friend virus in its long latent period (88 to 225 days) and in its inability to induce spleen foci. End-point dilution experiments suggested that a hitherto undescribed component of the Friend virus complex might be responsible for these late-appearing erythroid leukemias.

INTRODUCTION

Mурine leukemia viruses are capable of inducing lymphoid, myeloid, and erythroid leukemias (12). In some instances, a single virus isolate is able to produce more than one type of leukemia. This morphological variability has often been attributed to host factors, e.g., genetic constitution or the presence or absence of a specific target tissue, such as the thymus (9). Friend virus, however, is capable of inducing both erythroid and lymphoid tumors because it is a complex of more than one virus. One of these components, the replication-defective SFFV-F, produces erythroleukosis with a short latent period and, following i.v. inoculation, characteristic macroscopic splenic foci (1). Another component, LLV-F (4, 13), is replication competent and acts as the natural helper for SFFV-F (6). LLV-F has been isolated by passage through animals resistant to SFFV-F (4) and by end-point dilution (11, 13). The leukemic lymphocytes induced by LLV-F have been shown by immunofluorescence to be T-cells (2).

Recently, a virus capable of inducing ML in many strains of mice was isolated from a C57BL mouse which developed chloroleukemia following inoculation with LLV-F (10). The virus was closely related antigenically to LLV-F and arose either by selection of a preexisting variant in the LLV-F or by genetic recombination between the virus and the chloroleukemia cell.

We have inoculated LLV-F into BALB/c mice that were thymectomized when 3 to 5 days old and treated with ATS. These animals developed a leukemia that could be distinguished histologically, cytologically, and by immunofluorescence from the LL's produced by the same preparation of LLV-F in intact animals. In a preliminary report (3), we described the leukemia in T-cell-depleted animals as null cell. However, further morphological studies have shown it to be an erythroid neoplasm. Cell-free passages from animals with EL suggest that it is induced by an agent that is probably distinct from either LLV-F or SFFV-F.

MATERIALS AND METHODS

Mice. BALB/c mice were obtained from Simonsen Laboratories, Gilroy, Calif., and our own colony. They accepted skin grafts from one another and responded identically to Friend virus inoculation.

Virus. LLV-F, first isolated by us in 1966 (4), was used. This virus had been passaged 5 times in Scott-Russ rats and once in Fischer rats prior to passage in BALB/c mice. Virus pools were also prepared from the spleens of individual experimental leukemic mice by our standard method (5).

Tissue Transplantation. To establish tumors, leukemic mice were killed by cervical dislocation. Each spleen was removed aseptically, minced separately in phosphate-buffered saline, and inoculated into 3 to 5 syngeneic intact and/or thymectomized mice. Both newborn and young adult recipients were used; the former received 0.05 ml i.p., and the latter received 0.2 ml either s.c. or i.p. Cell viability was confirmed prior to inoculation by the trypan blue exclusion test. Only animals that developed splenomegaly within 40 days and showed transplantable leukemias histologically were considered positive.

Rabbit anti-mouse ATS was prepared against thymocytes from A/J mice in the laboratory of Dr. A. P. Monaco, Boston, Mass. NRS was obtained from New Zealand White rabbits and heat inactivated at 56°C for 30 min before use.

Indirect immunofluorescence was performed using the antisera and methods described previously (2).

Experimental Design. BALB/c mice of both sexes were thymectomized at 3 to 5 days of age. In one experiment, thymectomized mice received 0.1 ml of a 1 in 10 dilution of ATS i.p. on Days 8, 9, and 10 and 10².8 ID₅₀ of LLV-F i.p. on Day 9. In 2 other experiments, thymectomized mice were given 0.2 ml of undiluted ATS i.p. on Days 58, 60, 62, and 64 and 10³.1 ID₅₀ of LLV-F i.p. on Day 60. Intact untreated control animals of the same age were inoculated with virus simultaneously. In a subsequent experiment, mice were either thymectomized when 3 days old or given 0.05 ml of a 1 in 10 dilution of either ATS or NRS i.p. on Days 1, 3, and 5. All 3 groups died of leukemia between Days 15 and 30.

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were inoculated with $10^{2.3}$ ID$_{50}$ of LLV-F i.p. on Day 3. All mice were housed in plastic cages and were weaned when 3 to 4 weeks old. They were fed Purina laboratory chow with water ad libitum.

When the mice became terminally leukemic, hemoglobin measurements, WBC, and blood smears were made. They were then killed, and the thymuses, when present, as well as spleens, were removed aseptically for histology, cytology, and immunofluorescence. Virus pools were prepared from selected individual spleens, either immediately or after storage at $-70^\circ$. The completeness of all thymectomies was confirmed by histological examination.

RESULTS

The inoculation of LLV-F into intact and thymectomized ATS-treated (T-cell-depleted) mice produced 2 quite different diseases (Table 1). Intact mice developed leukemia composed of medium or large lymphocytes bearing the Thy 1.2 antigen with the leukemic cells located primarily in the thymus-dependent regions of the lymphoid organs (Fig. 1).

Virus-inoculated T-cell-depleted mice developed EL involving the red pulp of the spleen (Figs. 2 and 3) and sinusoids of the liver after a latent period of 88 to 225 days. The leukemic cells were small and round; the nuclei had finely stippled chromatin and 1 or 2 small nucleoli. The cytoplasm was scanty and amphophilic. Wright-stained imprints showed nuclei with finely granular chromatin and 1 or 2 rather inconspicuous nucleoli and deeply azurophilic cytoplasm (Fig. 4). Some cells matured to intermediate and late normoblasts. There were some areas of myeloid differentiation in the spleens of all animals; in 8 of 20 mice this was marked, and in 3 it predominated. In one mouse, the splenic sinusoids were full of undifferentiated hemopoietic stem cells. Blood counts were available in 18 of 20 T-cell-depleted virus-inoculated mice. These mice were generally anemic (mean hemoglobin of 9.2 g/100 ml; range, 6.0 to 14.6) and had a leukocytosis (mean of 48,300/cu mm; range, 9,200 to 100,000). Blood smears showed many smudge cells and erythroblasts. Cell suspensions prepared from the spleens of these mice showed neither the Thy 1.2 antigen nor detectable surface immunoglobulins.

EL's were successfully transplanted into 28 of 46 (61%) thymectomized recipients and 4 of 4 intact newborn recipients, but no tumors developed in the 39 intact recipients inoculated at 4 to 10 weeks of age. The transplantation of tissues from mice with a combination of EL and LL resulted in transplantable lymphocytic lymphomas in 22 of 62 (35%) intact adult recipients, but no EL's, indicating a marked difference in the transplantability of the 2 leukemias.

The effect of various degrees of T-cell depletion on leukemia induction was examined by inoculating mice with LLV-F following either either thymectomy at 3 days of age or neonatal treatment either with ATS or with NRS. The results (Table 2) showed partial suppression of the expression of LL in mice that were either thymectomized or given ATS. Treatment with NRS had no effect on leukemogenesis. Four T-cell-depleted animals in the original experiments were found at autopsy to have been incompletely thymectomized. All 4 had LL in the thymic remnant, and 2 had EL in their spleens. Eight mice thymectomized as newborns and given ATS on Days 58, 60, 62, and 64, but not inoculated with virus, showed no sign of leukemia when sacrificed at a mean age of 183 days (range, 98 to 248).

Results of our initial experiments were found at autopsy to have been incompletely thymectomized. Three had been given ATS and inoculated with virus at 9 days of age in accordance with the main experimental protocol.

### Table 1
Comparison of leukemia in intact and T-cell-depleted mice inoculated with LLV-F

<table>
<thead>
<tr>
<th>Animals inoculated as newborns</th>
<th>Intact mice</th>
<th>T-cell-depleted mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Latent period (days)</td>
<td>Thymic weight (mg)</td>
</tr>
<tr>
<td>9</td>
<td>78 (60—104)*</td>
<td>118 (20—350)</td>
</tr>
<tr>
<td>13</td>
<td>130 (88—174)</td>
<td>TA</td>
</tr>
</tbody>
</table>

* Mean; numbers in parentheses, range.

<table>
<thead>
<tr>
<th>Animals inoculated at 60 days of age</th>
<th>Intact mice</th>
<th>T-cell-depleted mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Latent period (days)</td>
<td>Thymic weight (mg)</td>
</tr>
<tr>
<td>7</td>
<td>208 (130—257)</td>
<td>200 (70—990)</td>
</tr>
<tr>
<td>14</td>
<td>146 (90—225)</td>
<td>TA</td>
</tr>
</tbody>
</table>

* Mean; numbers in parentheses, range.

### Table 2
Leukemia induced by LLV-F in newborn mice following various types of T-cell depletion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LL</th>
<th>EL</th>
<th>LL and EL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymectomy*</td>
<td>0</td>
<td>3</td>
<td>2*</td>
</tr>
<tr>
<td>ATS&quot;</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NRS&quot;</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Incomplete thymectomy and ATS&quot;</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* Mice were thymectomized at 3 days of age and inoculated i.p. with $10^{2.3}$ ID$_{50}$ LLV-F on Day 3.
" An additional mouse had ML.
* Intact mice inoculated i.p. with 0.05 ml of a 1 in 10 dilution of either ATS or NRS on Days 1, 3, and 5 and given $10^{2.3}$ ID$_{50}$ of LLV-F i.p. on Day 3.
* These mice were found to be incompletely thymectomized at the end of our initial experiments. Three had been given ATS and inoculated with virus at 60 days of age, and one had been given ATS and inoculated with virus at 9 days of age in accordance with the main experimental protocol.
To investigate whether specific viral factors were also involved in the induction of EL, mice were inoculated i.p. with 0.05 ml of cell-free extracts prepared from the spleens of 5 individual leukemic T-cell-depleted mice. Each pool produced a variety of leukemias; 6 mice developed LL, 4 had EL, and 1 had ML, while 21 had a combination of LL in the thymus and EL in the spleen.

These data suggested that a virus distinct from LLV-F might be producing the EL’s and an attempt was made to isolate it by end-point dilution (Table 3). Serial 10-fold dilutions of cell-free spleen extracts from an animal with EL were inoculated i.p. in 0.05-ml amounts into intact newborn mice. The results (Table 3) showed a progressive reduction in the number of mice developing both LL and EL so that at the highest dilution animals developed either LL or EL. These data, confirmed in a second experiment, strongly suggested the presence of a viral component in the original preparation of LLV-F that was responsible for this late-appearing erythroid, or occasionally myeloid, leukemia. In these experiments, which were done with virus from the third passage of the original isolates, the latent period ranged from 64 to 222 days (mean, 132 days).

To assure that this late-appearing EL was not merely a manifestation of Friend disease in T-cell-depleted mice, 15 newborn BALB/c mice were thymectomized; given 0.2 ml of ATS i.p. on Days 66, 68, and 70, and inoculated i.p. with 10^5-7 ID₅₀ of Friend virus on Day 68. Nineteen control mice received NRS and Friend virus. Both groups of mice were killed 21 days after virus inoculation, when all mice had marked splenomegaly; the mean spleen weight of the T-cell-depleted mice was 1880 mg (range, 1170 to 2740) and that of controls was 2470 mg (range, 1720 to 4470). Histologically, both intact and T-cell-depleted mice showed typical Friend disease. Although the distribution of the disease was similar to the EL described above, the individual cells in Friend disease had more abundant amphophilic cytoplasm, often with a clear perinuclear zone, and bigger, more vesicular nuclei with a single large nucleolus (Fig. 5). Immature myeloid cells were not observed in the spleens of T-cell-depleted mice inoculated with Friend virus. Friend virus is also capable of producing macroscopic erythroblastic spleen foci in mice when inoculated i.v. (1). To test the EL-producing isolate for this property, 14 intact young adult mice were inoculated i.v. with 0.4 ml of a cell-free spleen extract from a T-cell-depleted animal with EL. Ten were killed 12 days after inoculation, and 4 were killed at 28 days. None had splenomegaly or spleen foci.

**DISCUSSION**

Thymectomy is known to alter the morphological expression of many murine leukemia viruses. Some viruses that induce LL’s in intact mice have been found to induce ML in thymectomized animals (7, 8). Since the thymus is the target organ of most viruses producing LL, thymectomy has been presumed to effect leukemogenesis by depleting the host of susceptible lymphoid cells and allowing the expression of nonlymphoid leukemias. The possibility that the production of different leukemias in intact and thymectomized animals was due to the selective expression of different components from a mixture of leukemia viruses has not been well investigated.

In intact mice, LLV-F produces a T-cell LL (2). We have demonstrated that, in T-cell-depleted mice, our pools of LLV-F produce erythroid (and myeloid) leukemias. We have also shown that cell-free extracts of the spleens of erythroleukemic T-cell-depleted animals induced EL’s in intact mice, suggesting the presence of an EL-inducing viral agent distinct from LLV-F. The existence of a separate virus is also indicated by the apparent separation of the LL-inducing and EL-inducing activities achieved by end-point dilution.

There are several possible explanations for the presence of this ELV in our pools of LLV-F. It could be an attenuated form of SFFV-F which has persisted in our pools of LLV-F. We have shown, however, that the newly identified ELV differs from SFFV-F in several important ways. First, ELV does not induce spleen foci as does SFFV-F. Also, ELV has a much longer latent period than SFFV-F. In our end-point dilution experiments, long latent periods were seen even in animals receiving the largest dose of ELV (which we have estimated to be 10^3-6 ID₅₀). Finally, the EL described in these studies differs histologically from Friend disease produced by SFFV-F. Thus, we believe that ELV is not an altered form of SFFV-F. The difference between ELV and SFFV-F could be conclusively established by isolating the virus and showing that it is replication competent or, failing that, by showing nucleic acid sequence differences between ELV and SFFV-F (14).

It is possible that ELV is a passenger virus acquired during the multiple passages of our viral stocks through rats and mice. There is some evidence, however, that this virus has been present in our viral pools for a long time. Some years ago, we observed a number of mice which, following inoculation with one of our early pools of Friend virus, developed splenomegaly and EL after a latent period of approximately 200 days. Virus from these animals was successfully passaged and continued to induce EL with a similar long latent period. We now think that this virus may be the same as the ELV we described above and that ELV is probably not a recently acquired passenger virus or a new recombinant. ELV could, however, be a passenger or recombinant acquired early in the passage history of Friend virus, or it might have been a component of the original isolate of Friend virus. Further biochemical and immunological characterization of ELV must await isolation of the agent, which ideally should be done by cloning in vitro. However, we do not yet know if, like SFFV-F, it is replication defective or, indeed, if it can be cultured in vitro.

**ACKNOWLEDGMENTS**

The skilful technical assistance of Joan DeFehr and Constance Melcko is gratefully acknowledged.

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* A. H. Fieldsteel and P. J. Dawson, unpublished data.
REFERENCES

EL Induced by LLV-F

Fig. 1. LL involving the periarteriolar sheath in an intact mouse inoculated with LLV-F at 9 days of age. H & E, × 250.

Fig. 2. EL involving red pulp but sparing the periarteriolar sheath of the spleen of a T-cell-depleted mouse inoculated with LLV-F at 60 days of age. H & E, × 100.

Fig. 3. Same animal as in Fig. 2, showing cellular detail. Right, uninvolved malpighian body. H & E, × 500.

Fig. 4. Spleen imprint showing erythroid differentiation in a T-cell-depleted mouse inoculated with LLV-F at 60 days of age. Wright, × 1000.

Fig. 5. Friend disease in the spleen of a T-cell-depleted mouse inoculated with Friend virus at 70 days of age. H & E, × 500.
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