Leukemogenesis in Vitro Induced by Thymus Epithelial Reticulum Cells Transmitting Murine Leukemia Viruses

Martin Haas, Talia Sher, and Sara Smolinsky

Department of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

SUMMARY

The role of the thymus in induction of leukemia was studied in vitro. Cultivation of normal thymus cells on thymus epithelial reticulum cell monolayers that had been grown from radiation leukemia virus-induced leukemic thymuses rendered the thymocytes leukemic. C57BL/6 thymocytes were cultivated for 3 days on leukemic thymus reticulum monolayers, and 10^9 thymocytes were injected i.p. into young adult C57BL/6 mice. After 3 to 4 weeks all mice died of disseminated lymphatic leukemia. Mice given thymocytes that had been cultivated on thymus epithelial reticulum monolayers from normal mice did not develop lymphomas.

The leukemic thymus epithelial reticulum cells were shown to produce thymotropic as well as ecotropic and xenotropic radiation leukemia virus. (Thymotropic virus has affinity for thymus lymphocytes but does not infect fibroblasts.) The cells were brightly positive for murine leukemia virus group-specific antigen in immunofluorescence tests. Leukemic thymus epithelial reticulum cells produced ample infectious ecotropic virus in the culture supernatant, although the cells were negative in the XC syncytia test. Upon infection of mouse fibroblasts with ecotropic virus produced by the leukemic reticulum cells, XC syncytia were readily obtained. Thymocytes that were cultivated on leukemic thymus reticulum cells became positive for murine leukemia virus group-specific antigen and produced syncytia in the XC test. Thus, in vitro lymphomagenesis of the thymocytes that were cultured on leukemic thymus reticulum cells was associated with their infection with thymotropic and ecotropic radiation leukemia virus.

INTRODUCTION

The thymus plays an essential role in the induction of murine lymphatic leukemia. The spontaneous development of lymphatic leukemia in the high-leukemia-incidence AKR mouse strain is virtually abolished by thymectomy (21). Law and Miller (17) have shown that thymectomy prevents development of lymphomas following application of chemical carcinogens, and Kaplan (13) has shown that thymectomy reduces the incidence of radiation-induced lymphomas in C57BL/6 mice. Implantation of thymus grafts s.c. or i.r. in thymectomized animals restores the susceptibility of AKR mice to spontaneous lymphatic leukemia development (16) and restores the susceptibility of C57BL/6 mice to RadLV-induced lymphomas (14).

Law (16) has demonstrated that leukemias that developed in high-leukemia AKR grafts implanted into hybrid crosses of C3H and AKR thymectomized mice were of hybrid (host) origin. He postulated, therefore, that the thymus grafts increased the leukemia incidence in the host due to their reticular elements rather than their lymphoid part. Thymus grafts that were implanted in diffusion chambers were unable to reverse the inhibitory influence of thymectomy (23, 24). Moreover, Hays (10) has demonstrated that thymus grafts composed mainly of thymus epithelial reticular cells have the capacity to restore susceptibility to leukemogenesis, both spontaneous and virus-induced, in suitable thymectomized animals.

Thus, in high-leukemia mouse strains and in low-leukemia strains the thymus plays a central role in induction of leukemia, whether induced spontaneously, by virus inoculation, or by chemicals, or by physical agents. The specific role of the thymus in leukemogenesis may lie in the unusual properties of the thymus epithelial reticulum cells, which have been shown to be active immunologically (15, 25, 27) by virtue of the secretion of the thymic hormone thymosin (20). Thymus reticulum cells have also been shown to play a role in lymphopoiesis in the cortex of the normal (19) and leukemic thymus, probably by direct cell-to-cell contact between TR cells and thymic lymphocytes (22).

Elucidation of the involvement of TR cells in lymphogenesis has been complicated by the need to design complex in vivo experiments. The availability of in vitro pure TR monolayer cultures (1) made it possible to study the mechanism of the TR role in lymphomagenesis in vitro. Recently, in vitro lymphomagenesis on AKR TR monolayers was accomplished in our department (26). In the AKR system, high levels of Gross virus expression in all mouse tissues at all ages did not allow the study of virus involvement in the in vitro TR experiments. Therefore, we developed an in vitro leukemogenesis system in C57BL/6 mice. These mice are virus negative for the first 6 months of their life. X-irradiation induces leukemogenic RadLV in C57BL/6 mice, a virus that causes lymphomas and ultimate death of the host.
In studies comprising this paper normal C57BL/6 thymocytes were shown to undergo leukemogenesis after their in vitro incubation on top of RadLV-infected LTR cultures derived from RadLV-induced leukemic C57BL/6 mice. Thymocytes that were incubated on LTR cells but not on kidney reticulum, RadLV-infected B6 fibroblasts, or NTR cultures became infected with thymotropic RadLV and produced lymphatic leukemia in normal as well as in thymectomized recipient mice within 3 to 4 weeks. The interaction of LTR monolayers with normal thymocytes resulted in (a) the transfer of virus from monolayer to thymocytes, and (b) the genesis of potentially leukemic thymocytes, which may be due to the synergistic action of virus infection and specific differentiation signals transferred from LTR monolayers to the thymocytes. Cultivation of thymocytes on RadLV-producing monolayers other than TR cells did not render them leukemic.

MATERIALS AND METHODS

Mice. C57BL/6 female mice were used throughout this study. Mice were bred at the Weizmann Institute Animal Breeding Center. Thymomas were induced in 6- to 8-week-old mice by i.t. injection of RadLV (8). Thymectomized mice were operated on when they were 5 to 6 weeks old and were used 3 to 4 weeks after the operation. Thymectomized mice were always checked for total absence of a thymus when mice were moribund. Thymocytes were removed from normal 6- to 8-week-old mice and were dispersed steriley through a stainless steel grid and washed with phosphate-buffered saline as described before (3). Injection of cultivated thymocytes was done i.p.

Thymus Epithelial and Other Culture Methods. Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg kanamycin, 25 μg gentamycin, and 2.5 μg Fungizone per ml was used, except when otherwise indicated. Thymus epithelial reticulum monolayers were grown as described previously (1, 27). Cultivation of normal and leukemic TR monolayers was done identically. When Waymouth culture medium was used during the 1st day of TR plating, 100 units of penicillin and 100 μg of streptomycin per ml were added to the culture medium. Similarly, whenever TR cells were grown with Eagle's medium, the antibiotics used were penicillin and streptomycin only. The reticulum cells adhered to the Petri dishes and formed a complete monolayer by 6 to 9 days in culture. TR cells stained periodic acid-Schiff positive (27). When a few fibroblasts were found in the TR monolayers, these could be quantitatively removed by a 5- to 10-min treatment of the monolayer with 0.05% trypsin and 0.05% Versene at 37°C, followed by washing of the monolayer. This treatment left the TR monolayer intact. Short trypsin-Versene treatment did not damage the TR monolayers; experiments done with trypsinized TR cultures were identical to those done with nontryptsinized monolayers. In effect, the only way to remove the TR cells from the dishes was to scrape them gently with a rubber policeman, since trypsinization did not remove the TR cells.

Cultivation of Thymocytes on TR Monolayers. Thymocytes were cultivated on TR cells in Eagle's medium. Normally, 20 × 10⁶ thymocytes were added to a TR culture containing 1 to 2 × 10⁶ reticulum monolayer cells in a 60-mm tissue culture dish. In some experiments as many as 50 × 10⁶ thymocytes were cultivated per TR culture without apparent differences in experimental results. Removal of thymocytes at the end of the cultivation period was done by gentle shaking of the medium and 1 or 2 additional washes. TR cells were quantitatively retained on the dish by this method. During incubation of thymocytes on TR monolayers, thymocytes underwent autonsensitization (27).

Immunofluorescence Techniques. Indirect immunofluorescence of MuLV-gsa was done on acetone-fixed cells, as described by Hilgers et al. (11). The antiserum used was a rat anti-MuLV-gsa serum produced in Lewis rats by a RadLV-induced regressing rat lymphoma K127R (4). Its endpoint titer was 1:2048 to 1:4096, and it was used at a dilution of 1:64. Specificity for MuLV-gsa and other details about this antiserum have been described (5). TR cells were prepared for immunofluorescence either by scraping of the cells with a rubber policeman or by growing the cells on coverslips from the time of their 1st plating. Fibroblast cell lines were removed from the dishes by trypsinization and were washed 3 times with Earle's balanced salt solution without Ca²⁺ and Mg²⁺ before being put on immunofluorescence slides. Photography of immunofluorescent images was done on a Zeiss UV microscope with epi-illumination, a 200-watt HBO lamp, and Planapo × 40 oil immersion lens. Exposure was 30 to 90 sec on Kodak Highspeed Ektachrome film, exposed and developed as 400 ASA.

Virus Titration Methods. Virus titrations were performed using an infectious center assay. Dilutions of 24-hr culture supernatants containing 4 μg polybrene per ml (Aldrich Chemical Co., Milwaukee, Wis.) were inoculated on 24-hr cultures containing 5 × 10⁶ SC-1 or SIRC cells. The SC-1 mouse fibroblast cell line is nonrestrictive for mouse-tropic C-type ANA viruses (9); SIRC is a rabbit cornea cell line on which C57BL/6-derived xenotropic virus grows efficiently (18). Twelve hr after infection of SC-1 cells or 24 hr after infection of SIRC cells, the cells were dispersed for 5 min with 0.75% trypsin (9) and counted, and serial dilutions were made on standard 24-hr SC-1 or SIRC cultures growing on coverslips. Three or 4 days later the coverslips were fixed in acetone and stained for immunofluorescence of MuLV-gsa, and the number of infectious foci was determined under the UV microscope. In some experiments the infected SC-1 or SIRC cells were treated with 10 μg mitomycin C per ml for 2 hr and then washed before dispersion with 0.75% trypsin. Mitomycin C treatment did not change the results of the infectious center assays.

A cocultivation infectious center assay was used for the estimation of ectotropic and xenotropic viruses that were transmitted from TR cells to SC-1 or SIRC cells in cocultivations. SC-1 or SIRC cells (5 × 10⁶) were plated directly on a TR culture containing a counted number of cells. Twelve hr later SC-1 cells were removed by 0.05% trypsin-Versene; SIRC cells were removed 24 hr after plating on TR cells. The cells were then treated with 0.75% trypsin and plated as in the infectious center assay. No corrections were made for plating efficiency of infectious centers.

Reverse XC tests and cocultivations have been described.
RESULTS

1. Lymphatic Leukemia Induction by Thymocytes Incubated on TR Monolayers. TR cultures growing in 60-mm dishes typically contained 1 to 2 x 10⁶ monolayer cells. They were overlaid with 20 x 10⁶ young adult C57BL/6 thymus cells and incubated for 72 hr. Fig. 1 shows a phase-contrast micrograph of a typical TR culture 10 days after plating. The cells have a large central nucleus with a prominent nucleolus and marked cytoplasmic granularity. Less than 1 hr after addition of fresh thymocytes to the TR monolayer, major attachment of thymocytes to the monolayer cells occurred. Fig. 2 shows a LTR culture 2 hr after addition of 20 x 10⁶ thymocytes, in which the nonattached thymocytes were shaken off the plate in order to show that attachment of thymocytes took place to most of the LTR cells. Only 2 to 4 x 10⁶ thymocytes could be removed from the TR monolayer 2 hr after plating; the rest remained attached to the monolayer and could not be washed off. Major blast formation took place, and the enlarged thymocytes remained attached to the monolayer for 24 to 40 hr. At 48 to 72 hr most of the attached thymocytes could be removed from the monolayer by gentle washing with medium. Typically, after 72 hr, 4 to 6 x 10⁶ incubated thymocytes could be recovered; the rest had died during incubation, and a few remained attached to the TR monolayer. The recovered thymocytes were at least 90% viable by the trypan blue exclusion test.

One million thymocytes that had been incubated on LTR cultures were injected i.p. into young adult normal or thymectomized C57BL/6 mice. Table 1 shows the result of several such experiments. Most mice died within 3 to 4 weeks after the injection of thymocytes that had been incubated on LTR monolayers. Immunosuppression of the LTR-injected mice before the injection of incubated thymocytes did not appreciably shorten the latent period. The thymus was not necessary for the induction of lymphomas in the recipient mice, since thymectomy did not inhibit development of the lymphomas or prolong its latent period. The lymphomas that were obtained were processed for histology, and in all cases disseminated lymphatic leukemia was diagnosed. The spleen and lymph nodes were always involved, most cases showing major splenomegaly. Frequent spreading to the liver and kidneys was also observed. The thymus was leukemic in about one-half of the cases in which normal mice received injections. Some mice were sacrificed 2 weeks after injection of LTR-incubated thymocytes. In these mice, clear histological evidence of early leukemic changes could be observed at that time, which was 2 to 3 weeks before leukemic death normally occurred.

It was important to know whether the leukemic cells that gave rise to in vivo tumors were of donor-thymocyte origin or of LTR monolayer origin. Although no thymocytes were observed in the LTR monolayers, some leukemic cells could hide in the monolayer and be injected with the cultivated thymocytes to produce leukemia in the recipients. Therefore, control experiments were done in which we either seeded C3H thymocytes on C57BL/6 monolayers or where no thymocytes were seeded on the LTR monolayers. After a 3-day cultivation period the C3H thymocytes and whatever cells were present in the medium of the nonseeded monolayers were injected into C57BL/6 recipient mice. No lymphomas developed in these control mice (Table 2), indicating that the LTR monolayer was not the source of the leukemia-inducing cells.

Could reticulum monolayer cultures from healthy mice or RαLV-producing fibroblast cell lines induce thymocytes to become leukemic during in vitro cultivation?

In order to answer this question, epithelial reticulum monolayer cultures were grown from thymuses of healthy young adult mice. Thymocytes that were incubated for 3 days on such NTR cells were injected into B6 mice. No lymphomas

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pretreatment of recipient cells</th>
<th>No. of lymphomas/Av. latent period to leukemia death*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 None</td>
<td></td>
<td>9/9 27 ± 4</td>
</tr>
<tr>
<td>Thymectomy 4 wk before injection</td>
<td>10/10 24 ± 4</td>
<td></td>
</tr>
<tr>
<td>Immunosuppression by 400 R whole-body X-irradiation</td>
<td>5/5 29 ± 4</td>
<td></td>
</tr>
<tr>
<td>2 None</td>
<td></td>
<td>11/13 38 ± 8</td>
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<tr>
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<td></td>
<td>4/4 40 ± 8</td>
</tr>
<tr>
<td>3 Thymectomy</td>
<td></td>
<td>4/4 46 ± 7</td>
</tr>
</tbody>
</table>

*Latent periods are expressed in days after injection of incubated thymocytes

** Mean ± S.D.

Lack of lymphoma development by inoculation of thymocytes cultured on normal thymus epithelium and on other monolayers See legend to Table 1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Thymocytes cultured 72 hr on top of</th>
<th>No. of lymphomas/Av. latent period to leukemia death*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 NTR</td>
<td></td>
<td>0/10</td>
</tr>
<tr>
<td>2 Kidney reticulum from leukemic mouse</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>3 SC-i(LTR) ecotropic virus-producing cells</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>4 SIRC(LTR) xenotropic virus-producing cells</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>5 SC-i(LTR) + SIRC(LTR) virus-producing cell mixture</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>6 C3H thymocytes on C57BL/6 LTR monolayer</td>
<td>0/8</td>
<td></td>
</tr>
</tbody>
</table>

M. Haas et al. Distribution of Ectotropic and Xenotropic C-type RNA Viruses in Leukemogenic and Nonleukemogenic Virus-producing Radiation Leukemia Thymoma Cells, submitted for publication to the Journal of Virology.

See legend to Table 1.
were observed in these control experiments. We also incubated thymocytes on cell lines chronically infected with RadLV. Cell lines used were SIRC(LTR), the SIRC rabbit cornea cell line that was chronically infected with xenotropic RadLV from LTR cultures (see Section 4); SC-1(LTR), the ecotype indicator cell line chronically infected with ecotropic RadLV from LTR cultures (see Section 4); and B-6(RadLV), a C57BL/6 embryo cell line chronically infected with ecotropic RadLV. Thymocytes incubated on these RadLV-producing monolayers or on mixed cultures of these virus-producing cells did not produce lymphomas upon injection.

After establishing the specificity of in vitro lymphogenesis between LTR cells and normal thymocytes, we sought to elucidate the viral role in this interaction. Thus, in the remainder of this paper we describe experiments designed to investigate involvement of RadLV in this in vitro leukemogenesis.

2. Virus-infected Status of LTR Cells. The presence of replicating MuLV in LTR monolayer cells was investigated by immunofluorescent staining for MuLV-gsa, by radioactively labeling of culture supernatants and isolation of labeled virus, and by the XC syncytium test for MuLV. Indirect immunofluorescence using a rat anti-MuLV-gsa antiserum on fixed cells readily showed that 60 to 80% of LTR monolayer cells were MuLV-gsa positive. Fig. 3 shows that LTR cells had brightly fluorescent cytoplasms, indicating a high level of intracellular virus-associated antigen. Fig. 4 shows that NTR-cultured cells grown from healthy 8-week-old donors were negative for MuLV-gsa, as assayed by the immunofluorescence test. Indeed, NTR cells from 6- to 8-week-old animals have consistently been MuLV-gsa negative, whereas LTR monolayers registered MuLV-gsa positive.

Two 60-mm cultures of LTR monolayers and 2 cultures of NTR cells were labeled for 40 hr with [3H]glucosamine plus [14C]uridine, precursors of virus glycoproteins and virus RNA, respectively. Labeled culture supernatants were processed as described under "Materials and Methods," and virus was banded in sucrose gradients. Chart 1 shows that a peak of unidist- and glucosamine-labeled virus particles was found, at a density of 1.16 g/ml in the supernatant of the LTR-labeled monolayer. No such virus particles could be demonstrated in supernatants of NTR cultures. This suggested that MuLV virions were being produced by LTR cultures but not by NTR cultures, a result that corroborated the MuLV-gsa immunofluorescence data described above.

MuLV-gsa-positive LTR cultures that actively liberate virus particles were tested for syncytia production in an inverted XC test. The RadLV thymoma cells from which LTR monolayers were grown produced ample ecotropic RadLV when cocultivated with ecotype indicator cells (4). Therefore, the virus-producing LTR cells were also expected to produce XC syncytia in the inverted XC test. Nevertheless, LTR cells did not produce syncytia in the XC test, as is shown in Figs. 5 and 6. In an XC test of LTR cells, the large LTR nucleus with its granular cytoplasm and the surrounding XC cells may easily be mistaken for XC syncytia. As it is shown in Fig. 6, the XC cells did not overgrow the epithelial reticular cells, nor did they undergo the typical fusion resulting in syncytia formation. The XC syncytium-negative status of MuLV-gsa positive of LTR cells has been verified by immunofluorescent staining of LTR cells after these had been grown on coverslips and had been tested in an inverted XC syncytium assay, as shown in Fig. 6. Fig. 6 shows that MuLV-gsa-positive LTR cells did not produce syncytia even though the cells were actively synthesizing virus. This suggested initially that LTR cells produced a xenotropic RadLV, a virus known to be inactive in XC syncytia induction and positive by MuLV-gsa immunofluorescence staining. As will be shown below, LTR cells actively produced infectious ecotropic virus, which was an XC syncytia nonproducer virus when growing in LTR cells but which produced ample syncytia upon infection of mouse fibroblasts.

3. Infection of Thymocytes with RadLV during Cultivation on LTR Monolayers but not on NTR Monolayers, Ecotropic Virus-producing Mouse Fibroblasts, or Xenotropic Virus-producing SIRC Cells. It was shown above that healthy thymocytes that had been incubated on LTR monolayers were leukemic when injected into young C57BL/6 mice. It was also shown that the LTR cells actively produced RadLV. Therefore, we investigated whether, during cultivation, virus transmission took place from LTR monolayers to the suspended thymocytes. Normal thymocytes that were cultivated on LTR monolayers for 72 hr were tested by immunofluorescence for expression of MuLV-gsa. Forty to 70% of these thymocytes became brightly fluorescent (Fig. 7). In contrast, thymocytes that were incubated on NTR monolayers or on the ecotropic or xenotropic virus-producing cells B6(RadLV), SC-1(LTR), SIRC(LTR), or SC-1(LTR) + SIRC(LTR) did not become infected with virus as assayed by immunofluorescence of MuLV-gsa. By definition, virus infection of the thymocytes that were cultivated on LTR monolayer cells represents a thymotropic (having affinity for thymic lymphocytes) interaction. This suggests that LTR cells are infected with a thymotropic virus (8) that is transmitted to thymocytes upon their cultivation on top of the
LTR monolayer. The lack of infection of thymocytes by RadLV-ecotropic and xenotropic component viruses during the cultivation of thymocytes on SC-1(LTR) or SIRC(LTR) virus-producing cells strengthens the case for the involvement of a thymotropic virus in the LTR-thymocyte in vitro interaction.

To further study whether the MuLV-gsa-positive thymocytes had become virus infected during their cultivation on LTR monolayers, LTR-cultured thymocytes were plated on top of XC cultures, and syncytia were counted 3 days after fixing and staining of the cells. Titration of the syncytia obtained showed that 9 to 16% of the thymocytes plated gave rise to XC syncytia. Thus, thymocytes that were cultivated on LTR cultures became infected with a syncitium-producing virus. Whether, in addition to thymotropic virus, ecotropic and xenotropic viruses were also transmitted from the LTR cells to the cultivated thymocytes is not yet clear.

4. Infectious Ecotropic and Xenotropic MuLV Synthesized by LTR Cell Cultures. Two different assay techniques were utilized for titrating ecotropic and/or xenotropic viruses produced by the TR cultures. One technique consisted of a conventional infectious center assay for the titration of supernatant virus. The other technique was a “cocultivation infectious assay” which estimated infectious virus that was transmitted during cocultivation of TR cells and fibroblast indicator cells (see “Materials and Methods”). Ecotropic virus was assayed on SC-1 cells, and xenotropic virus was assayed on SIRC cells. Table 3 shows that infectious ecotropic virus could be recovered from LTR supernatants, but no infectious xenotropic virus was found. NTR cultures released neither ecotropic nor xenotropic infectious virus into the culture medium.

A cocultivation infectious virus assay was devised in order to learn whether any xenotropic virus of LTR cells might be titrated as well. In short, this semiquantitative assay functioned as follows. SC-1 or SIRC cells (5 x 10⁶) were plated directly on top of the reticulum monolayer culture. After 12 or 24 hr the cells were selectively removed with trypsin-Versene without removing the reticulum cell monolayer, and an infectious center assay was performed with the cocultivated SC-1 and SIRC cells. Table 4 shows that, by using this assay, high titers of ecotropic and xenotropic virus could be recovered from LTR monolayers. Low titers of xenotropic virus have also been obtained from some NTR monolayers.

Ecotype and xenotype cells that had been infected with ecotropic and xenotropic virus by cocultivation on LTR monolayers were subcultured twice until the viruses had spread evenly in all susceptible cells of the cultures. These infected cells, designated SC-1(LTR) and SIRC(LTR), became 80 to 100% brightly MuLV-gsa positive and produced infectious ecotropic or xenotropic virus, respectively. Supernatant virus titers in SC-1(LTR) and SIRC(LTR) cultures were of the order of 10⁴ infectious ecotropic virions per ml and 2 x 10⁶ infectious xenotropic virions per ml, respectively. The infected cells, SC-1(LTR) and SIRC(LTR), were infected by cocultivation on top of LTR cells, which transmitted both the ecotropic and xenotropic viruses. Consequently, either infected cell line might produce a mixture of both these virus classes. We checked this and found that SC-1(LTR) cell cultures did not produce any xenotropic virus, nor did SIRC(LTR) cells produce any ecotropic infectious virus. Thus, these cocultivation-infected cell lines produced only ecotropic or xenotropic virus, respectively.

SC-1(LTR) and SIRC(LTR) cells were tested for their ability to produce XC syncytia in an XC test. A number of the virus-positive SC-1(LTR) cells and virus-positive SIRC(LTR) cells were plated and overlaid 24 hr later with 5 x 10⁴ XC cells. Three days later the plates were fixed and stained, and XC syncytia were scored. Every SC-1(LTR) cell produced a syncytium, whereas, as expected, the xenotropic virus-infected SIRC(LTR) cells produced no XC syncytia at all. Fig. 8 shows XC syncytia that were produced by SC-1(LTR) cells in the XC test. The LTR cells that actively produced infectious ecotropic virus and were brightly MuLV-gsa positive did not produce XC syncytia in the XC assay (Fig. 6), whereas, upon transmission of this virus to the fibroblast cell line SC-1, every cell produced a clear XC syncytium. The state of the replicating ecotropic virus in TR cells was therefore different from its state in fibroblasts as was indicated by the inability of infected LTR cells to produce XC syncytia.

**DISCUSSION**

Furth et al. (2) have shown that cells harvested from 20-day in vitro cultures of suckling rat thymus were associated with the development of lymphoma at the graft site when inoculated into neonatally thymectomized virus-infected rats. Hays (10) has shown that epithelial remnants prepared from neonatal thymus were effective in reconstituting the depleted lymphoid tissues and in facilitating a return to normal immunological function of neonatally thymectomized mice. Wekerle et al. (27) and others (15) have shown...
that TR monolayer cultures were able to induce immune competence of T-lymphocytes.

Pure thymus epithelial reticulum monolayers have been available in this laboratory for some time (1). Also, it has been possible to demonstrate in vitro leukemogenesis in high-leukemia AKR mice (26). As most AKR tissues and cells are virus infected from birth, viral analysis of the in vitro leukemogenesis experiments was difficult. Therefore, in vitro TR lymphomagenesis was attempted in the C57BL/6 system. The mouse strain is virus negative up to the age of about 6 months, although it gives rise to potently leukemogenic RadLV after X-irradiation.

In the present study it was found that lymphomagenesis of healthy thymocytes resulted when these were cultured on TR cultures taken from RadLV-induced leukemic mice. C57BL/6 mice that received syngeneic thymocytes cocultivated on LTR monolayers developed disseminated lymphatic leukemia that could be detected histologically 2 weeks after injection and killed the animals in 3 to 5 weeks. Induction of leukemia by the LTR-cultured thymocytes was independent of the host thymus, indicating that the thymus-independent steps associated with leukemogenesis had taken place in vitro. The latent period of this lymphoma induction was short compared to that of leukemia induced by RadLV (7). The short latent period and the thymus independence of this lymphoma induction suggest that it was not due merely to the transfer to RadLV from the LTR cultures to the recipient mice, but was due to inoculation of leukemic cells.

Culturing of healthy thymocytes on LTR monolayers gave rise to leukemic cells but thus far did not trigger in vitro transformation of the thymocytes, as defined by the establishment of immortal leukemic cell lines. It is not clear what factors or cofactors are present in vivo that allowed multiplication of the injected leukemic cells and that were absent from the cultures. Another important question is whether the in vivo lymphoma cells actually represented the in vitro cultivated thymocytes. In the absence, so far, of a suitable thymocyte genetic marker in congenic C57BL/6 strains, we resorted to showing that LTR monolayer cultures that were seeded with C3H thymocytes did not produce any leukemias in C57BL/6 mice. This excluded the possibility that leukemic cells hidden in the LTR monolayer and carried along with C3H thymocytes were the ones responsible for leukemia induction in the recipients. In addition, it has been shown recently in this laboratory that in AKR mice, in vitro leukemogenesis of thymocytes cultured on AKR LTR monolayers was caused by lymphomagenesis of the cocultured normal thymocytes. The thy-1 genetic marker was used in the analysis (26). In this system the lymphomas were shown to carry the marker of the cultivated thymocytes rather than that of the reticulum monolayer or of the recipient mice.

What is the mechanism of this in vitro-induced lymphomageneses? Is it of viral origin, and what is its relation to leukemogenesis in vivo?

TR cells grown from RadLV-induced thymomas were found to be infected with thymotropic virus (thymocyte-infectious virus) in addition to high-titer eco- and xenotropic viruses. Consequently, the LTR cells were MuLV-gsa positive. Infectious ecotropic but not xenotropic virus has been found in culture supernatants, whereas high titers of both viruses were found to infect suitable indicator cells by cell-to-cell contact during cocultivation.

Thymocytes that were cultured on the virus-producing LTR cells became chronically infected with virus which, by definition, is thymotropic. Some 10 to 20% of the MuLV-gsa-positive thymocytes also produced XC syncytia.

Virus found in the thymocytes that had been cultivated on LTR monolayers might have been induced in the thymocytes by the LTR cells or, alternatively, it might be the result of infection with LTR virus during cocultivation. Our results suggest that this 2nd alternative is tenable, as normal thymocytes have not been observed to induce any mouse-tropic virus when cocultivated with a variety of monolayer cells (4), although they do produce xenotropic virus during cocultivation. Induction of mouse-tropic virus in thymocytes has never been observed to occur, even when they were induced to blasts in specific cocultivation combinations.

TR cultures or kidney reticulum cultures did not transform incubated thymocytes to a leukemic state, nor did virus-infected fibroblasts induce a leukemogenic state in thymocytes incubated on them. Moreover, thymus epithelial reticulum cells were distinct from kidney reticulum cells and from mouse embryo cell lines in their ability to induce differentiation of thymus-dependent lymphocytes (27). LTR cells were also distinct in their ability to efficiently infect thymocytes with MuLV, as was shown in these studies. Neither MuLV-infected C57BL/6 fibroblasts (syngeneic with the thymocytes) nor SC-1(LTR)-infected mouse cells could efficiently infect the cultivated thymocytes with MuLV. Indeed, if infection of thymocytes did occur upon their cultivation with the MuLV-producing fibroblasts, it was not detectable by immunofluorescence.

Thus, it seems plausible that the combined action of virus infection of the thymocytes and specific (cell-to-cell) differentiation signals that have been shown by others to act in the reticulum cell-thymocyte interaction is responsible for the specific lymphomagenesis that we have observed in vitro. Exposure of thymocytes to ecotropic and/or xenotropic RadLV, even to such viruses isolated directly from the active LTR cultures in SC-1(LTR) and SIRC(LTR) cells, did not by itself produce a leukemic state in the thymocytes, nor could NTR cells induce this state in the absence of the virus, as we have shown.

We have shown previously (3, 6) that the leukemogenic virus of the RadLV complex was thymotropic (i.e., it preferentially infected thymus lymphoid cells). We have also shown (4) that thymoma cells that produce only leukemogenic thymotropic virus produce mouse-tropic and xenotropic virus upon their cocultivation with permissive indicator cells. The thymotropic virus is defective for fibroblasts (6) and semidefective upon infection of lymphoid cells (M. Haas, unpublished data). Thus, the simplest explanation of the in vitro lymphomagenesis data and the virologica data presented in this paper is that TR cells are permissive for both thymotropic and ecotropic RadLV. The latter virus may serve as helper agent for the semidefective thymotropic virus. By virtue of the permissiveness of TR cells for thymotropic and ecotropic RadLV, these cells may facilitate the transmission to thymus lymphoid cells of this thymotropic-
leukemogenic agent. Whether the unique lymphocyte differentiation ability of TR cells plays a role in this virus lymphomagenesis is suggested but not known.

The state of the ecotropic virus in LTR cells was distinct, as these cells did not produce XC syncytia. Upon transmission of the ecotropic virus from LTR cells to SC-1 mouse embryo fibroblasts, the LTR-ecotropic virus produced XC syncytia efficiently. Ecotropic MuLV that are syncytia non-producers have been described before by Hopkins and Jolicoeur (12). These authors also suggested that the syncytia nonproducer virus variant could give rise to a syncytia producer virus by passage through the proper host cell, which is what is suggested by our experiments with ecotropic RadLV from LTR cells. Another, alternative explanation for the syncytia nonproducer state of the infected LTR cells is that these cells may secrete a factor that is inhibitory to XC syncytia formation. The finding that XC cells do not overgrow NTR or LTR cells makes this a tenable explanation.

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REFERENCES


2. Furth, J., Kunii, A., Iiochim, H., Sanel, F. T., and Moy, P. Parallel Observations on the Role of the Thymus in Leukemogenesis, Immuno-


4. Haas, M. On the Necessary and Sufficient Properties for Leukemogenic-


6. Haas, M., and Hilgers, J. In Vitro Infection of Lymphoid Cells by Thryo-

7. Haran-Ghera, N. Latent Period in Leukemia Induction by the Radiation


10. Hays, E. F. The Role of Thymus Reticular Cells in Viral Leuke-


13. Kaplan, H. S. Influence of Thymectomy, Splenectomy and Gonadectomy on the Incidence of Radiation-Induced Lymphoid Tumors in Strain C57/


21. McEndy, D. P., Boon, M. D., and Furth, J. On the Role of the Thymus, Spleen, and Gonads, in the Development of Leukemia in a High Leuke-


25. Pyke, K. W., and Gelfand, E. W. Morphological and Functional Matura-


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Martin Haas, Talia Sher and Sara Smolinsky


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