Quantitation, in Vitro Propagation, and Characterization of Preleukemic Cells Induced by Radiation Leukemia Virus

Eitan Yefenof, Sergio Epszteyn, and Moshe Kotler

Lautenberg Center for General and Tumor Immunology [E. Y., S. E.] and Department of Molecular Genetics [S. E., M. K.], Hebrew University-Hadassah Medical School, Jerusalem 91 010, Israel

ABSTRACT

Intrathymic (i.t.) inoculation of radiation leukemia virus into C57BL/6 mice induces a population of preleukemic (PL) cells that can progress into mature thymic lymphomas upon transfer into syngeneic recipients. A minimum of 10^6 PL thymic cells are required to induce lymphomas in the recipient. Most of the individual lymphomas developed in mice which were inoculated with cells of a single PL thymus, derived from different T-cell precursors. PL thymic cells could be grown in vitro on a feeder layer consisting of splenic stromal cells. Growth medium was supplemented with supernatant harvested from an established radiation leukemia virus-induced lymphoma cell line (SR4). The in vitro-grown PL cells were characterized as Thy-1+, CD4+, CD8− T-cells, most of which expressed radiation leukemia virus antigens. Cultured PL cells were found to be nontumorigenic, based on their inability to form s.c. tumors. However, these cells could develop into thymic lymphomas if inoculated i.t. into syngeneic recipients. A culture of PL cells, maintained for 2 mo, showed clonal T-cell receptor arrangement. Lymphomas which developed in several recipient mice upon injection with these PL cells were found to possess the same T-cell receptor arrangement. These results indicate that PL cells can be adapted for in vitro growth while maintaining their preleukemic character.

INTRODUCTION

Promoter insertion, alternative splicing, and enhancement of cellular oncogene expression are mechanisms by which retroviruses lacking oncogenes can induce a malignant disease (1). A prerequisite for the occurrence of such transformational events is the integration of a provirus at the vicinity of a specific oncogene within the host cell genome. Proviral integration into a unique DNA site occurs rather infrequently; therefore, it can be predicted that virus-derived malignancy is a rare event (1–11). This prediction is supported by the long latent period preceding the development of tumors induced by slow oncogenic retroviruses, the clonal origin of these tumors, and their infrequent appearance (12).

RadLV is a slow leukemogenic retrovirus which induces clonal thymic lymphomas after a latent period of 3 to 6 mo following i.t. inoculation of susceptible C57BL mice (13–15). However, potentially leukemogenic cells appear in the thymus as soon as 10 days post inoculation (16–18). These cells, termed PL cells, can be identified by their ability to progress into mature lymphomas when transferred to syngeneic recipients (19–21). The early emergence of PL cells implies that RadLV exerts its transforming potential shortly after inoculation and long before the appearance of an overt lymphoma. Thus, initial steps in RadLV leukemogenesis are frequent and occur in multiple preleukemic host cells. This PL cell population therefore undergoes a tumor progression process involving the selection of a single PL cell that eventually expresses a fully malignant phenotype (22).

In a previous study (23), we demonstrated that 1 to 2 days following RadLV inoculation about one-third of the thymic lymphocytes express viral envelope antigen. The proportion of virus-positive cells in the thymus decreases gradually and reaches a level of 1 to 2% at wk 3 post virus inoculation. Viral antigen-expressing cells persist at low frequency until the appearance of an overt lymphoma, when most of the thymic cells are replaced by virus-positive T-lymphocytes.

In the present study, we determined the minimal leukemogenic dose of PL thymocytes from RadLV-inoculated mice which is required to induce lymphomas in syngeneic recipients. In addition, thymic cells derived from RadLV-inoculated mice were successfully cultured. In vitro and in vivo analysis of the cultured cells suggested that they represent a population of RadLV-infected T-lymphocytes which can develop into clonal lymphomas when inoculated i.t. into syngeneic mice.

MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from the animal house facilities of the Hebrew University-Hadassah Medical School.

Cell Lines. Dr. M. Haas kindly provided 136.5, a RadLV-induced T-cell lymphoma cell line (24). C41 is a clone of a helper T-cell lymphoma induced by RadLV (25). Additional cell lines used in this study were YAB3, YAB9, and SR4; all were established in our laboratory from RadLV-induced thymic lymphomas. The lines were maintained in RPMI-1640 medium containing 10% FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

Virus Inoculation. Highly leukemogenic RadLV was harvested from 136.5 cell cultures at intervals of 24 h (24). The virus was concentrated 100-fold by clearing the supernatant at 10,000 × g for 15 min at 4°C in a Sorvall superspeed centrifuge, followed by centrifugation at 19,000 rpm for 2.5 h at 4°C. The pellet was resuspended with PBS at 1/100 of the initial volume, aliquoted, and stored at −70°C. Forty μl of the thawed virus preparation were injected i.t. into 6-wk-old C57BL/6 mice as described (26).

PL Cell Culture. Spleen cells of C57BL/6 mice were irradiated with 3500 rads and placed in Petri dishes to form a feeder monolayer of splenic stromal cells. A lymphoid cell suspension was prepared from thymuses of RadLV and mock infected C57BL/6 mice 3 wk post inoculation. The stromal cell monolayer was overlaid with thymocytes at a concentration of 2 × 10^6 cells/ml in DMEM supplemented with 10% FCS, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 0.5 μM 2-mercaptoethanol, and 1% of a solution containing 5.8 g of L-arginine, 0.6 g of folic acid, and 3.6 g of L-asparagin per liter. In some cultures 25% of the medium was replaced by supernatant derived from SR4 cells (SR4-SN). Viability of cultured lymphoid cells was determined by trypan blue exclusion every 1 to 3 days. The medium was changed twice weekly by replacing half of the cultured cell suspension with fresh medium.

Cytofluorimetry. Cells (5 × 10^6) were washed 3 times with Hanks' balanced salt solution containing 0.2% sodium azide and 1% FCS (washing buffer). The cells were incubated with 100 μl of the indicated mouse monoclonal antibody diluted in PBS for 30 min at 4°C. The

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: RadLV, radiation leukemia virus; PL, preleukemic; i.t., intrathymic; FCS, fetal calf serum; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; IL, interleukin; SSC, standard saline citrate; MoAb, monoclonal antibody.
cells were washed twice in washing buffer and incubated with 100 μl of conjugated fluorescein isothiocyanate: goat anti-mouse immunoglobulin (Sigma) diluted 1:20 in PBS for 30 min at 4°C in the dark. The cells were washed twice as before, fixed in 1% formaldehyde in PBS, and analyzed in a fluorescence-activated cell sorter (FACS IV; Becton and Dickinson).

The following monoclonal antibodies were used: anti-L3T4 (GK1.5), Thy1.2 (HO-13-4), and anti-Ly2.2 (Ho2.2), obtained from the American Type Culture Collection; and anti-RadLV monoclonal antibody (2F10) produced in our laboratory.

Transfer of PL Cells. PL thymocytes from a single mouse (3 wk post inoculation) or cells from a single preleukemic culture were inoculated i.t. into 5 to 10 syngeneic mice. The mice were checked for lymphoma development for at least 5 mo. It has been previously shown that lymphomas arising in such recipients are of donor origin (15, 16, 23).

Tumorigenesis Assay. Cells (10⁷) were injected s.c. into syngeneic mice. Palpable tumors were identified, and their diameter was measured every second day using an engineer’s micrometer (Mitutoyo, Japan).

Thymidine Incorporation Assay. PL cultures were cultivated in 96-well microtiter plates (Nunc) with medium containing SR4-SN. Various concentrations of anti-IL-4 antibodies (11B11) or anti-IL-2 antibodies (S4B6.2), kindly provided by Dr. W. E. Paul, were added to the culture. The cultures were incubated for 48 h and then pulsed with 1 μCi of [³H]thymidine (Amersham) for 6 h. [³H]Thymidine incorporation was determined by measurement in a scintillation counter.

DNA Preparation and Hybridization. High-molecular-weight DNA was isolated by a modification of the proteinase K/phenol/chloroform method of Gross-Bellard et al. (27). DNA was digested with restriction enzymes and separated on 0.8% agarose gels. The DNA was acid depurinated before denaturation and transferred to nitrocellulose filters. The filters were hybridized with 2 × 10⁶ cpm/ml of nick-translated probe in hybridization mix that contained 50% formamide, 10% dextran sulfate, 1.5 x SSC, 5x Denhardt’s solution, and denatured salmon sperm DNA (100 μg/ml) at 42°C (28). The filters were washed with 0.1x SSC and 0.1% sodium dodecyl sulfate at 65°C.

DNA Probe. The probe was isolated from the 86T5 plasmid (29), kindly provided by Dr. M. Davis. This plasmid contains a segment of DNA coding for the T-cell receptor β-chain (T₇), which had been isolated by cleavage with EcoRI. The DNA fragment was isolated by electrophoresis on an agarose gel and labeled by nick translation as described (30).

RESULTS

Quantitation of Preleukemic Cells in RadLV-injected Mice. In order to determine the minimal leukemogenic dose of PL thymocytes, different numbers of cells derived from a single RadLV-injected mouse were inoculated i.t. into syngeneic recipients. All the mice which were inoculated with 10³ or 10⁴ thymic cells developed thymic lymphomas and died within 65 to 77 days (Fig. 1). Mice receiving 10⁴, 10⁵, or 10⁶ cells died as well, but only after a delayed latency of 75 to 108 days (Fig. 1). One of 5 mice inoculated with 10² PL thymus cells died 118 days post inoculation. The rest of the mice survived at least 180 days post inoculation, at which time the experiment was terminated.

Clonality of Lymphomas Developed in Recipient Mice. DNA was extracted from primary lymphomas developed in mice inoculated with different doses of PL thymocytes. The PL thymocytes were derived from a single mouse which had been inoculated with RadLV 3 wk earlier. The DNA preparations were subjected to Southern blot analysis using a probe specific for the T-cell receptor chain (29). Fig. 2 shows that the germ line T₇ gene is detected in a 6.7-kilobase fragment following digestion by PvuI. The polyclonal T-cell population in the thymus had essentially the same pattern as the germ line. A RadLV-induced lymphoma C-41 appeared with two additional bands belonging to the rearranged allele, indicating its clonality. Three tumors that developed in mice inoculated with 10⁴ thymic cells derived from a single PL mouse arose from at least 2 different T-cell clones (Vi4w41 versus Vi4w42 and Vi4w43). All the tumors which developed in mice inoculated with 10⁴, 10⁵, or 10⁶ cells had a particular T₇ rearrangement and therefore originated from different clones.

In Vitro Propagation of PL Cells. In an attempt to culture PL cells, suspensions of thymic lymphocytes were prepared from a PL thymus 3 wk post RadLV inoculation. The cells were placed over an irradiated monolayer of splenic stromal cells, and the fraction of viable cells was determined every 1 to 3 days. Thymocytes derived from a mock-infected mouse were cultured under similar conditions as a control. Fig. 3 shows that no continuous cell growth was detected when the cells were cultured in complete DMEM supplemented with FCS alone. However, addition of 25% SR4-SN sustained continuous growth and proliferation of thymic cells derived from a PL mouse. Thymic cells derived from the mock-infected mouse did not grow under the same culture conditions. The PL cultures could be maintained at cell densities of 1 to 5 × 10⁶ cells per ml with medium replacement twice weekly. Proliferation of the lymphoid cells was dependent on close contact with the monolayer of adherent stromal cells (Fig. 4). Removal of lymphoid cells to flasks lacking a feeder layer resulted in complete cell death within 48 h (not shown). Replacement of the SR4-SN by IL-2 (50 units/ml), IL-4 (200 units/ml) (Genzyme), or combinations thereof did not support cell proliferation of cultured cells (results not shown). However, addition of the anti-IL-4 MoAb 11B11 to cultures containing SR4-SN abolished DNA synthesis in the cells (Fig. 5), indicating that IL-4 is an essential but not sufficient factor in promotion of preleukemic cell growth. Antibodies that block IL-2 activity did not affect the proliferative response of the PL cells to SR4-SN (Fig. 5).

Surface Characteristics of PL Lines. SR4 and YAB3 as well as many other RadLV-induced lymphomas express the T-cell markers Thy1.2 and L3T4 (Fig. 6) (31). PL7, PLSR, LS1R, and L73 are PL lines derived from different RadLV-inoculated mice. These cells also expressed Thy1.2 and L3T4, indicating that they belong to the CD4⁺, CD8⁻ T-cell lineage (Fig. 6). RadLV-induced lymphomas express virus antigens on their cell surface, as indicated by their interaction with antibodies directed against the virus envelope glycoprotein (Fig. 6) (31). The four PL lines tested were also reactive with the anti-viral
RadLV-INDUCED PRELEUKEMIC CELLS

Fig. 2. T4 rearrangement in a series of thymic lymphomas (Vi4w) developed in mice which were inoculated with the indicated number of PL cells. DNA from kidney (G. L.), thymus (Thymus), C-41, and Vi4w primary lymphomas was digested with PstII and Southern blotted using the 86T5 probe. Markers were prepared by HindIII cleavage of λ-DNA and indicated in kilobase (kb) units.

![Image of Southern blot](image_url)

Fig. 3. Proliferation of PL thymocytes in culture. Thymic lymphocytes from RadLV or mock-infected mice were cultured on monolayers of splenic stromal cells. ⊗, PL thymocytes + SR4 SN; □, mock-infected thymocytes + SR4-SN; △, PL thymocytes; ○, mock-infected thymocytes; ▼, PL thymocytes without splenic stromal cells.

glycoprotein antibody (Fig. 6), suggesting virus production in the cells.

Tumorigenicity of the PL Cell Lines. Inoculation of RadLV-induced lymphomas cells s.c. into syngeneic mice results in progressive growth of local tumors, which eventually kill the host (32). The diameter of tumors developed from YAB3 and YAB9 cells increased progressively until the fifth week post inoculation, when all the inoculated mice died. Mice inoculated with SR4 cells developed initial tumors at a similar rate, but on the third week post inoculation, when the tumors reached a diameter of 3.5 to 4 mm, all the mice died. No palpable tumors developed in mice inoculated s.c. with 10⁶ PL7, LSR1, L73, and PLSR cells which have been grown in culture for 2 mo (results not shown). Mice inoculated s.c. with the PL cells remained alive for at least 6 mo post inoculation when the experiment was terminated. On the other hand, when C57BL/6 mice were inoculated i.t. with 10⁵ cultured PL7 or PLSR cells, thymic lymphomas developed and killed all the mice within 4 to 8 wk post inoculation (Fig. 7). Similar results were obtained when the mice were inoculated with 10⁶ PL cells (results not shown).

Clonal Analysis of Lymphomas Derived from PL Lines. As shown in Fig. 2, lymphomas arising in individual recipients inoculated with freshly explanted PL thymic cells derive from...
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Fig. 5. Thymidine incorporation into PL cells cultivated in the presence of 25% SR4-SN. Anti-IL-4 or anti-IL-2 monoclonal antibodies were added to the cultures at the indicated concentrations, and 48 h later, the thymidine uptake was determined.

![Thymidine incorporation graph](image)

Fig. 6. Cytofluorimetric analysis of leukemic (YAB3 and SR4) and PL (PL7, PLSR, LSR1, and L73) cells. A, Thyl.2; B, L3T4 (CD4); C, Ly2.2 (CD8); and D, 2F10 (RadLV envelope glycoprotein).

![Cytofluorimetric analysis](image)

**DISCUSSION**

In the present study, we found that thymic lymphomas are induced upon inoculation of 10^3 PL thymocytes into syngeneic recipient mice. Our previous findings demonstrated that, at this stage of the preleukemic latency, about 2% of the lymphocytes in the thymus are infected by the virus (23). Thus, it can be estimated that, in order to develop lymphoma, the thymus has to be inoculated with at least 20 virus-bearing T-cells. The fact that 100 PL thymic cells are not leukemogenic, although such a dose contains some virus-infected cells (1, 2), is in line with our previous conclusion that RadLV-induced leukemogenesis involves selection of a single malignant precursor cell from a polyclonal population of RadLV-infected PL cells. Indeed, T₆ rearrangement analysis revealed that individual lymphomas which arise following inoculation of either limiting or exceeding doses of PL thymic cells were derived from different T-cell clones. Thus, when the preleukemic cell population remains within the thymus of the virus-inoculated host, a clonal lymphoma will ultimately arise due to the selection of a single PL cell that acquires a malignant phenotype. However, if the PL cell content of the thymus is split into several recipients, pro-
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Progression of leukemogenesis occurs independently in each recipient and yields clonal lymphomas derived from different PL precursors.

The existence of PL cells in mice inoculated with RadLV has been determined based on their ability to develop lymphomas when injected into susceptible hosts (13–21). A more direct study of PL cells was hampered by the difficulty of isolating those cells due to their low number in the infected thymus (23). Recently, a procedure was reported by Haas et al. (33, 34) describing the in vitro propagation of factor-dependent early T-cell lymphomas induced by irradiation or RadLV. Such cells can proliferate in culture when cocultivated with adherent splenic stromal cells. We therefore attempted to cultivate PL cells from thymuses of RadLV-inoculated mice on monolayers of splenic stromal cells, albeit without success. However, addition of SN from a RadLV-induced lymphoma (SR4) supported the proliferation of thymic cells derived from a RadLV-inoculated thymus. Since SR4 cells constitutively secrete IL-4 (to be published), we assumed that IL-4 stimulated the T-cell growth in the thymic-stromal cell cultures. However, replacement of SR4-SN by IL-4 was not sufficient to maintain lymphoid cell proliferation in the coculture (to be published). On the other hand, cell growth in cultures containing SR4-SN was inhibited by 11B11 MoAb which specifically neutralizes IL-4 activity (35). We therefore concluded that PL lymphoid cells respond to IL4, but additional factor(s) present in the SR4-SN are required for their continuous growth. Alternatively, interaction of RadLV particles released from SR4 cells with the thymic-derived lymphocytes may also be required to maintain their in vitro growth. Virus-mediated growth signals might be transmitted following the specific binding of RadLV to CD3 and CD4 complexes expressed on T-cells, as demonstrated by O’Neill et al. (36).

Explantation of thymocytes from mock-infected mice to cultures of stromal adherent cells supplemented with SR4-SN did not lead to lymphoid cell growth. This result indicates that the proliferating cells in culture, initiated with thymocytes of virus-inoculated mice, were RadLV-infected PL cells. We therefore proceeded to characterize these cells, in comparison with several cell lines grown in vitro, derived from mature RadLV-induced lymphomas. Both PL cell lines and the mature leukemic lines express Thy1.2 and L3T4 T-cell differentiation markers. Expression of viral envelope antigen was detected in more than 90% of the malignant lymphoma cells. In the PL cultures, virus-positive cells were detected at a frequency of 50 to 70%, although these lines were initiated with thymocytes of which no more than 2% were infected by the virus (22, 23). These results indicate that the virus-infected T-cells were enriched in the culture, either because the noninfected cells were gradually disappearing, or because they were infected by the replicating virus in vitro.

PL cells in RadLV-inoculated mice have been previously defined as lymphocytes lacking malignant competence but capable of progressing into mature lymphomas in the thymic milieu. Can the cultured, virus-positive T-lymphocytes be considered RadLV-induced PL cells? This question was investigated by comparing the malignant properties of established RadLV-induced lymphomas with cultured PL cells. A direct

Fig. 2. Tc rearrangement in a series of thymic lymphomas developed in mice inoculated i.t. with PLW8 cells which have been cultured for 2 mo. DNAs from kidney (G.L.), thymus (Thy), C-41, PLW8, and six distinct LW8 lymphomas (LW81 to LW86) were digested with HindIII (a), Pvull (b), and EcoRl (c); Southern blotted; and hybridized with 86T5 probe. Markers are as in Fig. 2.
parameter of malignancy is the ability of the cells to grow and form local tumors when inoculated s.c. into syngeneic hosts (31). This property is shared by all the RadLV-infected cell lines established from mature thymic lymphomas. However, none of the PL lines could induce palpable tumors when injected s.c., suggesting a nonmalignant character of the cells.

The capability of RadLV-induced PL cells to progress and develop into thymic lymphomas when inoculated into thymuses of susceptible hosts is well documented (13–21). We therefore injected the in vitro-propagated PL lines i.t. into C57BL/6 mice and found that all of them developed thymic lymphomas with a latency period similar to that required for the appearance of lymphomas in the primary hosts. This observation suggests that cultured PL cells resemble normal thymic lymphocytes phenotypically and are not malignant. However, they can be distinguished from normal T-cells by their ability to grow in vitro, by their expression of RadLV antigens, and by possessing a leukemogenic potential, which could be manifested only when inoculated into a susceptible thymus.

Having established that in vitro-propagated cells have a preleukemic character, we determined their clonal origin. Several independent lymphomas which developed in a group of mice that were inoculated i.t. with cells of a PL line had an identical T<sub>r</sub> rearrangement, suggesting a common T-cell precursor. The T<sub>r</sub> rearrangement shared by these lymphomas was also detected in the cultured PL cells themselves. These results suggest that the explantation of PL cells and their adaptation to in vitro growth result in selection of a single premalignant clone which dominates the culture. This clone is the common progenitor of all the lymphomas induced in recipient mice. Thus, some initial steps of the multistage tumor progression, i.e., selection of a dominant premalignant clone, may also occur while the PL thymic cells are cultured in vitro.

REFERENCES

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