Clonal Analysis of Radiation Leukemia Virus-induced Leukemic and Preleukemic Murine Cells

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ABSTRACT

Clonality of radiation leukemia virus (RadLV)-induced thymic lymphomas was determined by detection of rearrangements in the genetic locus coding for the β chain of the T-cell receptor (TRA). Unique Tα rearrangements were detected in four of six lymphomas. Two of the Tα-rearranged thymic lymphomas and two in which such a rearrangement was not detected had a biallelic deletion of Cβ1. With an anti-RadLV monoclonal antibody it was found that 1–2 days after virus inoculation more than one-third of the cells in the thymus were infected by the virus. The frequency of virus-positive cells gradually declined and persisted at 1–2% until the appearance of a clonal lymphoma at which time virtually all the cells in the thymus were virus positive. Transfer of thymocytes from a single, preleukemic mouse 21 days post-virus inoculation into several adoptive recipients resulted in donor-type thymic lymphomas in the majority of the mice. Tα rearrangement analysis revealed that these lymphomas were clonal and derived from different potentially leukemic (preleukemic) cells in the thymus of the donor mouse. Eleven of 15 lymphomas had a biallelic deletion of Cβ1. These results suggest that clonal, RadLV-induced thymomas are selected from an oligoclonal, RadLV-infected preleukemic T-cell population.

INTRODUCTION

Oncogenic retroviruses can be divided into two groups: (a) acute oncogenic viruses such as Abelson (1) or murine sarcoma virus (2) that harbor sequences of transforming oncogenes (of cellular origin) in their RNA. These viruses have the capacity to transform cells in vitro and their inoculation in vivo results in tumor induction after a short period of 2 to 3 weeks; (b) slow oncogenic viruses, such as Gross/AKR (3) and Moloney leukemia virus (4). These viruses lack an onc gene and cannot transform cells in vitro, but they induce tumors in susceptible hosts after a long period of several months. Many of the slow oncogenic viruses cause a long term viremia in the inoculated host and the tumors they induce are generally of a monoclonal origin (5–8). The “promoter insertion model” elucidated in avian leukosis virus-induced leukemias (9) has been suggested as a possible explanation for the long preleukemic latency of this and other retrovirally induced leukemias. According to this model a nontransforming slow acting virus can infect a multiple population of target cells and randomly integrate into the cellular genome. Occasionally the proviral DNA integrates at a location at which its promoter/enhancer regions activate the transcription of a c-oncogene in a single cell (c-myec in avian leukosis virus) resulting in clonal leukemia. However, this model has not been established as the sole basis for preleukemogenesis of other virally-induced leukemias in which neither a specific viral integration site nor a common rearrangement in c-onc is detectable (10–12). Moreover, some slow acting retroviruses induce potentially neoplastic cells shortly after their inoculation and long before an overt tumor has appeared (13). The existence of such preneoplastic cells has been shown in C57BL/6 (B6) mice inoculated with RadLV. This virus, originally isolated from a radiation-induced lymphoma, can induce a high incidence of T-cell leukemias in adult B6 mice after a latency of 3 to 5 months (14). However, as early as 2 weeks post-virus inoculation preleukemic cells, identifiable by their ability to develop into donor-type leukemias in syngeneic recipients, appear in the thymus and bone marrow (15, 16).

The appearance of these preleukemic cells at the initial phase of latency implies that their ability to develop into leukemic cells is determined shortly after they have been infected by the virus but that the manifestation of their oncogenic potential is dependent on a postinfection process of progression and selection.

In the present study we analyzed the clonal nature of RadLV-induced preleukemic and leukemic cells and concluded that RadLV leukemogenesis involves selection of clonal leukemias out of a polyclonal preleukemic cell population.

MATERIALS AND METHODS

Animals. C57BL/6 (B6) mice obtained from the animal facilities of the Hebrew University-Hadassah Medical Center were used.

Cells and Cell Lines. Lines 136.5 and 127 are RadLV-induced T-cell lymphoma lines provided by Dr. M. Haas and Dr. N. Haran-Ghera, respectively. C-41 is a clone of a helper T-cell lymphoma induced by RadLV (17). We also used additional lymphoma cell lines freshly induced by RadLV as indicated in “Results.” The lines were maintained in RPMI 1640 containing 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Virus Inoculation. Highly leukemogenic RadLV was derived from 24-hour-old supernatants of a RadLV-transformed cell line (TIM.1) (18). Virus was quantitated by measuring the reverse transcriptase activity in the supernatants according to the method of Ross et al. (19). Mice were inoculated i.t. as described elsewhere (20) with 40 µl containing 0.04 reverse transcriptase units of RadLV as determined in the reverse transcriptase assay.

Limiting Dilution Cloning. Cells were seeded at 0.1 cell/well in 20-µl microtest tissue culture plates. Plates were wrapped with aluminum foil and incubated at 37°C and 5% CO2. Proliferating clones were transferred into 96-well U-shaped microtiter plates on days 5–10 and cultured for another week. The cloned lines were then transferred to 75-cm2 tissue culture flasks and maintained in culture as above.

Transfer of Preleukemic Cells. Male mice were inoculated i.t. with RadLV as described. Three weeks later the mice were sacrificed and thymocytes derived from a single mouse were inoculated i.t. into 4–5 female mice (10 cells/mouse). The mice were followed for 3–5 months and those which developed thymic leukemias were sacrificed.

Cytofluorometry. Thymus cells were incubated with the anti-RadLV monoclonal antibody 4D6 (diluted 1:5) for 30 min at 4°C. 4D6 is an IgG2a monoclonal antibody directed against the envelope glycoprotein (M, 70,000 glycoprotein) of RadLV. 4D6 is secreted by an hybridoma established in our laboratory following fusion of P3x63Ag8.653 myeloma cells with splenic lymphocytes derived from a C57BL/6 X BALB/C 

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3 The abbreviations used are: RadLV, radiation leukemia virus; i.t., intrathymically; MoAb, monoclonal antibody.
c F1 mice immunized with RadLV. The cells were then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Hyland Laboratories, Los Angeles, CA) for 30 min at 4°C. Control samples were treated directly with the fluorescein isothiocyanate conjugate. The cells were washed twice and then analyzed on a fluorescence-activated cell sorter (FACS-III; Becton and Dickinson) to determine fluorescence intensity and frequency of positive cells.

Purification of DNA. Total cellular DNA was purified by means of sodium dodecyl sulfate-Pronase digestion, extraction with phenol chloroform, and RNase treatment. The aqueous phase was precipitated with 2.5 volumes of ethanol and 100 mM sodium acetate at -20°C.

Restriction Enzyme Digestion and Analysis of DNA. DNA samples were digested with EcoRI or PvuII restriction endonuclease under conditions stipulated by the suppliers (Amersham, BRL). DNA fragments were separated by electrophoresis on 0.8-1% agarose gels. The fractionated DNA was then denatured and transferred to nitrocellulose filters by blotting in 20 M NaCl 10 mM Na2(PO)4-0.1 mM disodium EDTA, pH 7.0, essentially as described by Southern (21). In some experiments the radioactive probe was removed from the nitrocellulose blots by 3 h incubation at 70°C with 10 mM Tris-HCl (pH 7.5) during which the buffer was changed several times. The radioactive clearance was checked by exposing the filters for 2 days after which they were rehybridized with a second probe.

Isolation of Specific Probes. Plasmids 86T5 (22) and RBL5-17 (23) were kindly provided by Dr. M. Davis and Dr. T. Mak, respectively. These plasmids contain a segment of T which had been isolated by electrophoresis on agarose gels and labeled by nick translation as described (24).

RESULTS

Thymocyte Reactivity with 4D6 Antibody following RadLV Inoculation. Cytocentrifuged profiles of thymocytes stained with 4D6 MoAb are shown in Fig. 1. At 30 h after virus inoculation 35% of the cells in the thymus reacted with the antibodies (Fig. 1A). The frequency of positive cells dropped to 12% after 10 days (Fig. 1B) and 30 days after inoculation less than 2% of the cells reacted with 4D6 (Fig. 1C). Fig. 1D depicts analysis of thymic lymphoma cells developed in a mouse 3 months after virus inoculation. Essentially 100% of the cells reacted strongly with the anti-virus antibodies.

Fig. 2 summarizes the frequencies of 4D6 MoAb-stained cells in thymuses of RadLV-inoculated mice. Virus positive cells were first detected 10 h after RadLV inoculation; their percentage increased to a maximum of 35% 1 day later which persisted for 3 days more. The frequency of positive cells gradually leveled off to 1-2% by the 3rd week and remained constant for at least 6 weeks.

Tβ Gene Rearrangement as a Marker of Clonality. Fig. 3 shows a partial map of the Tβ genetic locus in the mouse as elucidated by Chien et al. (25) and Gascoigne et al. (26). The locus is composed of 2 adjacent genes for the constant region (Cβ1 and Cβ2), each having a separate cluster of D and J genes. It contains multiple restriction sites for EcoRI and PvuII and therefore clonal rearrangements of the Tβ region can be detected in Southern blots of DNA digested by one of these enzymes and hybridized with a Tβ specific probe.

In Fig. 3A DNA derived from kidney cells (germ line), thymus cells (representing a polyclonal T cell population), and C-41 cells (representing a clonal T-cell population) were digested by either EcoRI or PvuII and analyzed by Southern blot hybridization with the 86T5 probe. EcoRI digestion of kidney-derived DNA revealed 2 bands 2.2 and 9 kilobases long, corresponding to Cβ1 and Cβ2, respectively. Bands of the same size were detected in an EcoRI digest of DNA derived from a polyclonal T-cell population (thymus). When DNA of C-41 was analyzed an additional band of about 8 kilobases was detected, which represents a unique rearrangement of Tβ in this T-cell clone.

PvuII digestion of both germ line and thymus DNA resulted in the appearance of a single 6-kilobase band which appears to contain 2 fragments of equal size, one of Cβ1, and the other of Cβ2 (Fig. 3B). A clonal rearrangement of Tβ in C-41 cells was identified by the appearance of two additional bands 8 and 5 kilobases long, respectively.

Analysis of Clonality of RadLV-induced Thymic Lymphomas. In Fig. 4 we analyzed the clonality of several RadLV-induced lymphomas. DNA digestion by both PvuII (Fig. 4A) and EcoRI (Fig. 4C) revealed bands of 86T5 hybridization in Y11, 41, and 127 cells, which were unique for each tumor. In addition, the 2.2-kilobase band that corresponds to the 5' region of Cβ1 in the EcoRI digests was missing in Y1, Y2, Y10, and Y11, indicating a biallelic deletion of Cβ1 in these cells.

The radioactive probe in Fig. 4, A and C, was removed by melting and washing the nitrocellulose filters which were then
rehybridized with the RBL5-17 probe that selectively hybridized to Cβ1. A new 2.7-kilobase band appeared in blots of EcoRI-digested germ line and thymus DNA that corresponds to the 3' region of Cβ1 which is not detected by 86T5. Both the 2.2- and the 2.7-kilobase fragments were missing in Y1, Y2, Y10, and Y11 but could be detected in C-41 and 127 cells. The single 6-kilobase fragment of PvuII-digested DNA detected with 86T5 was also detected with RBL5-17 in germ line DNA and in DNA from thymus, C-41, and 127 cells. Again, RBL5-17 did not hybridize with blots of Y1, Y2, Y10, and Y11 cells.

Since no new fragments of Tβ rearrangements were detected in Y1, Y2, and Y10, we examined the possibility that the tumors are composed of a polyclonal T-cell population. Fig. 5 shows Southern blots of 4 clones derived from Y1 by limiting dilution cloning (Y1.1–Y1.4). Both EcoRI and PvuII digestions revealed that all the clones and the parental line shared an identical 86T5 hybridization band that corresponds to Cβ2 and a deletion of the 2.2-kilobase fragment containing Cβ1. Limiting dilution cloning of Y2 and Y10 presented similar results which indicate that the tumors are of a clonal nature (not shown).

Analysis of Clonality of Lymphomas Developed in Recipient Mice. Three weeks after i.t. inoculation of RadLV in male B6 mice, thymus cells were transferred to 6 irradiated B6 females (10' cells/mouse). Most of the mice developed donor-type thymomas as confirmed by the presence of the male Y-chromosome identified in a C-banding karyotype analysis. In Fig. 6 each of the series designated S, K, P, and R represents a number of thymic lymphomas developed in recipients that had been inoculated with thymus cells of a single donor. EcoRI (Fig. 6A) and PvuII (Fig. 6B) digestion of DNA derived from the “S” tumors demonstrated Tβ clonal rearrangements in S1, S2, and S3. Those of S1 and S2 were similar and that of S3 was unique. No novel bands of T were detected in S4 but this tumor had a biallelic deletion of Cβ1. These results suggest that the 4 tumors were derived from at least 3 different clones of donor T-cells.

Likewise, it was found that the tumors of the K and P series are derived from at least two T-cell clones and that tumors of the R series are derived from at least 3 clones. 86T5 hybridization to blots of DNA digested by EcoRI also revealed a biallelic deletion of Cβ1 of 1 of 4 “S” tumors, 2 of 3 “K”
CLONALITY OF RadLV-INDUCED LEUKEMIAS

Fig. 6. Tβ rearrangements revealed by 86T5 in thymic lymphomas of mice that have been adoptively transferred with thymocytes of RadLV-inoculated donors. S, K, P, and R, series of lymphomas developed in individual mice inoculated with cells of a single thymus; A and D, blots of an EcoRI digest; B and E, blots of a PvuII digest. In C and F the nitrocellulose filters of B and E, respectively, were melted and rehybridized with RBL5-17. M, markers; G.L., kidney; and 41.C-41; Kb, kilobases.

tumors, and all the “P” and “R” tumors (a total of 11 of 15).

Washing and rehybridization of the blots as depicted in Fig. 6, B and E, with RBL5-17 confirmed the deletion of the DNA fragments corresponding to Cβ1 and Jβ1 in 11 tumors. In addition it revealed a Cβ1-containing fragment of a PvuII digest in K5, which was larger than that of the germ line. This may be the result of a deletion of the PvuII restriction site located at the 3′ region of Jβ1 (Fig. 3B). The other 2 fragments (6-kilobase germ line and 3.4-kilobase PvuII-digested K5 DNA) (Fig. 6B) that did not hybridize to RBL5-17 (Fig. 6C) apparently derived from the second chromosome in which Cβ1 had been deleted.

These results indicate that preleukemic cells in the thymus 3 weeks post-virus inoculation are oligo- or polyclonal.

DISCUSSION

In the present study we analyzed the clonality of RadLV-induced leukemic and preleukemic cells. Since tumors induced by this virus are of T-cell origin we were able to elucidate their clonal nature by identifying rearrangements in the Tβ genetic locus coding for the β chain of the T-cell receptor. This approach has been used successfully to study clonality of human T-cell lymphomas and leukemias (27–29) and was adopted to the murine system in the present study. This has been done by DNA digestion with either EcoRI or PvuII and Southern blot hybridization with a complementary DNA probe (86T5) that is homologous to the Jβ1–Cβ1 gene cluster and cross-hybridizes with Cβ2 as well.

Comparison of kidney, thymus, and C-41-derived DNA established the reliability of the assay. A clone of RadLV-induced lymphoma (C-41) had a unique Tβ rearrangement as indicated by the appearance of novel 86T5 hybridization bands. However, blots of the polyclonal thymus cell population, which contain different Tβ rearrangements, resembled the germ line pattern, which was represented by kidney-derived DNA. Thus, in a polyclonal T-cell population the size distribution of different restriction fragments of Tβ cannot be detected by Southern hybridization with the 86T5 probe which enables distinction between clonal and polyclonal T-cell populations.

We then analyzed several RadLV-induced thymomas for clonality and found clonal rearrangements in 4 of 6 tumors. Quite unexpectedly, the 2.2-kilobase fragment that corresponds to Cβ1 could not be detected in 4 tumors, 2 of which appeared to have a Tβ rearrangement and 2 possessing the germ line Cβ2-containing fragment of 9 kilobases. A deletion of Cβ1 may not be sufficient to determine clonality because the possibility that the tumor is composed of a Cβ1-deleted oligoclonal T-cell population cannot be ruled out. We therefore isolated several clones of the Cβ1-deleted Y1 thymoma and tested them for clonality. Assuming that our inability to detect Tβ rearrangements in Y1 is due to its polyclonal nature, we expected to detect unique Tβ rearrangements in individual Y1-derived clones. However, this was not the case as 4 clones of Y1 appeared to be identical with their parental tumor. These results suggest that Y1 is a clone derived from a T cell in which Cβ1 has been deleted from both chromosomes and the Jβ2-Cβ2 did not rearrange or that such a rearrangement did occur but cannot be detected following DNA digestion with EcoRI and PvuII.

Potentially leukemic (preleukemic) cells in B6 mice appear as early as 2 weeks after RadLV inoculation and long before overt thymomas develop (30, 31). Thus, the commitment of the RadLV-infected preleukemic cell to become malignant is determined shortly after virus inoculation, but manifestation of leukemia requires a tumor progression process which takes place over a period of several months. Since RadLV-induced thymomas are monoclonal, we attempted to examine the clonal nature of their preleukemic progenitors. Using an anti-RadLV MoAb we found that as early as 15 h post-virus inoculation a substantial proportion of the cells in the thymus reacted with the antibody and the frequency of virus-positive cells increased to 35% after 1–2 days. The cell reactivity with the antibody apparently reflects viral infection and budding of virus particles rather than passive adherence of the virus to the cell membrane because during the first 10 h after virus inoculation i.t. no positive cells could be detected, and this timing corresponds to the kinetics of retroviral infection, integration, and replication (32). The percentage of virus-positive thymocytes dropped gradually to less than 2% 3 weeks after virus inoculation and persisted at this low frequency until the appearance of an overt lymphoma, when the thymus is repopulated by lymphoma cells of a monoclonal origin which are all infected by RadLV. These results suggest that RadLV infects a large polyclonal T-cell population shortly after it is inoculated i.t. and that the majority of the virus-infected cells are subsequently eliminated.

In addition, we attempted to examine the clonal nature of the preleukemic cell population by adoptively transferring thymus cells of a male mouse inoculated with RadLV 3 weeks earlier into several syngeneic female recipients. Most of the mice developed thymic lymphomas traced to the donor by identification of the male Y-chromosome (33). Primary tumors developing in a series of recipient mice inoculated with thymus cells of a single donor were analyzed for clonality by Southern blotting. The nonidentical bands of Tβ rearrangements that could be detected in individual lymphomas derived from a single preleukemic thymus suggested that although mature RadLV-induced thymic lymphomas are clonal, 2 weeks post-virus inoculation the thymus contains several clones of T-cells containing a leukemogenic potential. Such clones can progress independently and develop into mature, clonal lymphomas when inoculated into different recipients.

The analysis of Tβ in these tumors also revealed that the 2.2-kilobase EcoRI restriction fragment which corresponds to Cβ1 was not detected in 11 of 15 thymomas. This indicates that the majority of the RadLV-induced thymomas tested had a biallelic
deletion of Cβ1. Previous studies have shown a deletion of the entire Tβ genetic locus in suppressor T-cell hybridomas (34, 35). However, De Santis et al. (36) found that RadLV-induced suppressor T-cell lymphomas have a deletion in both Cβ1 alleles and rearrangements in their Jβ2–Cβ2 alleles. It is tempting to speculate that the high frequency of Cβ1 deletions in RadLV-induced thymomas reflects an advantageous progression of RadLV-infected T-cell clones that suppressor activity into mature lymphomas. The disappearance of the majority of the RadLV-infected T-cells 1–2 weeks after virus inoculation may be the result of thymus cell turnover and/or generation of antiviral immunity which can selectively eliminate virus-infected cells. The appearance of anti-RadLV cytotoxic T-lymphocytes in resistant mouse strains following inoculation with the virus has been widely documented (18, 37, 38). It has also been shown that the activity of such cytotoxic T-lymphocytes in susceptible mouse strains is abrogated by suppressor T-cells that emerge following inoculation of RadLV (18, 38, 39). Thus, selection of suppressor T-cells infected by RadLV may be a step in leukemia progression which enables escape of preleukemic cell clones from immunosurveillance. This might also explain the ease by which RadLV-induced suppressor T-cell lines are established (40, 41).

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REFERENCES

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