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Mechanism of T-cell lymphomagenesis: Transformation of growth-factor-dependent T-lymphoblastoma cells to growth-factor-independent T-lymphoma cells

(cell propagation in thymic microenvironment/tumor progression/chromosomal abnormalities)

MARTIN HAAS*†, AMNON ALTMAN‡, ELLEN ROTHENBERG§, MARK H. BOGART¶, AND O. W. JONES†¶

*Department of Biology and †Cancer Center, University of California at San Diego, La Jolla, CA 92093; ‡Scripps Clinic and Research Foundation, La Jolla, CA 92037; §Division of Biology, California Institute of Technology, Pasadena, CA 91125; and ¶Division of Genetics, University of California Medical School at San Diego, La Jolla, CA 92093

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ABSTRACT **In a previous paper we described the induction by x-irradiation or radiation-induced leukemia virus-inoculation of two classes of lymphoid T-cell neoplasms: The first class, designated T-cell lymphoblastoma (TCLB), consists of growth-factor-dependent eudiploid cells that home to the spleen and give rise to splenic tumors on injection into syngeneic mice; the second class, designated T-cell lymphoma (TCL), consists of growth-factor-independent aneuploid or pseudodiploid cells that give rise to local tumors at the site of subcutaneous injection. This paper describes the generation of a family of growth-factor-independent aneuploid or pseudodiploid TCL cells after the injection into the thymus of growth-factor-dependent diploid TCLB cells. In contrast to the donor TCLB cells, the resulting TCL cells could be cloned in semisolid medium, produced local tumors at the site of subcutaneous injection, and proliferated in a growth-factor-independent fashion *in vitro*. The induced growth-factor-independent TCL cells were chromosomally and phenotypically unstable and continued to evolve both *in vivo* and *in vitro*. After propagation in the thymus, the cells often showed stable translocations in addition to the evolving aneuploidy. We propose that the chromosome abnormalities induced during the proliferation of growth-factor-dependent TCLB cells in the thymus constitute a general mechanism by which neoplastic cells progress from growth-factor dependency to independency.**

The mechanism of evolution of T-cell lymphomas (TCL) within the thymus of systemically irradiated or radiation-induced leukemia virus (RadLV)-inoculated C57BL/6 (B6) mice is as yet unknown. Some models suggest that in the preneoplastic thymus there exists a continuing regenerative hyperplasia of cells leading to neoplasia, with the subsequent crowding out of the normal lymphocytes by neoplastic lymphoblasts (1). This mechanism is based on the existence in treated mice of potentially neoplastic cells that would evolve to become neoplastic TCL cells by propagation in the thymus. Potentially neoplastic cells have been shown in systemically irradiated (2, 3) as well as in RadLV-inoculated mice (4) using a transplantation bioassay in which hematopoietic cells taken from treated mice were injected into genetically marked recipients.

While one can thus show the presence of potentially neoplastic cells long before lymphoma development, the location of such cells in irradiated mice is a matter of contention. Some investigators have detected potentially neoplastic cells in the thymus although not in the bone marrow of irradiated mice (3); others have found preleukemic cells primarily among bone marrow cells but not in the thymus (5). Furthermore, few properties of potentially neoplastic cells are known (6, 7) because they have neither been grown nor otherwise isolated from the putative organs of their *in vivo* residence.

We have propagated and cloned in culture a class of T lymphoblasts from the spleens and lymph nodes of irradiated or RadLV-inoculated mice long before these had developed a thymic lymphoma (8). These neoplastic cells (T-cell lymphoblastoma; TCLB) precede the appearance of thymic lymphomas by 6–10 weeks. The isolation of these TCLB cells (8) suggests that they, or cells possessing similar properties, may be precursors to a class of autonomous neoplastic TCL cells. TCLB cells are distinct from thymic TCL cells in the following ways: (*i*) They are growth-factor dependent, autostimulating [producing their own growth factor(s)], and eudiploid; and (*ii*) they do not form colonies in soft agar unless the cultures are supplemented with exogenous conditioned medium. When injected *in vivo*, TCLB cells give rise to splenic tumors. Their tumorigenicity depends on the fact that they home to the spleen; they do not induce tumors at the site of subcutaneous inoculation. These characteristics of TCLB cells have been stable through prolonged propagation *in vitro* in mass culture, through cycles of single-cell cloning, and through repeated *in vivo* (splenic) passage.

In contrast, x-ray- or virus-induced thymic TCL are often monoclonal and are composed of chromosomally abnormal, growth-factor-independent (i.e., autonomously growing) cells. TCL cells can be quantitatively cloned in soft agar without added growth factors and produce tumors at the site of subcutaneous inoculation *in vivo*. TCL cells are often trisomic for chromosome 15.

On the basis of their growth-factor-dependent proliferation, their stable eudiploid karyotype, as well as their lack of cloning in methyl cellulose and their organ-dependent proliferation *in vivo*, TCLB cells can be classified as "benign" cells. In contrast, thymic TCL cells possess the characteristics of "malignant" cells, which grow autonomously and often metastasize.

After systemic x-irradiation or RadLV inoculation of B6 mice, growth-factor autonomous thymic lymphomas appear with a latency of 3–5 months, a latency that has not been satisfactorily explained. We surmised that cells possessing characteristics similar to the growth-factor-dependent TCLB cells might constitute natural precursors to a class of thymic TCL cells. If so, then continuing proliferation of TCLB cells in the thymus might lead to the selection of cells transformed into growth-factor-independent TCL cells. Here we report that intrathymic inoculation of cloned TCLB cells did induce rapidly growing thymic lymphomas composed of donor cells

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Abbreviations: TCLB, T-cell lymphoblastoma; TCL, T-cell lymphoma; RadLV, radiation-induced leukemia virus.

that had been drastically altered by proliferation in the thymus. Cells of thymic lymphomas that were derived after intrathymic inoculation of TCLB cells could be cloned in semisolid medium, grew into local tumors at the site of subcutaneous injection and were aneuploid in contrast to the eudiploid, growth-factor-dependent donor TCLB cells. After proliferation in the thymus, the cells often showed chromosomal translocations in addition to the evolving aneuploidy. Such lymphomas could develop from injection of an average of one TCLB cell. TCLB cells that had been propagated in the thymus thus had acquired many of the properties of radiation- or virus-induced thymic TCL cells, suggesting that propagation of immortalized T-cell blasts in the thymic microenvironment is necessary and may be sufficient for the generation of growth-factor-independent TCL cells.

MATERIALS AND METHODS

Tumor Cells. The induction of lymphomas by irradiation or virus-inoculation of B6 mice has been described (8). Establishment of TCLB and TCL cell lines in culture, as well as their cloning in semisolid medium, has also been described (8).

Intrathymic Inoculation of Cloned TCLB Cells. Mice injected intrathymically with TCLB cells develop rapidly growing thymic tumors. From the time enlarged thymuses could be palpated (thymus size, 300–400 mg; 16–18 days after injection) to the time of death (thymus size, 2–3 g), only 2–3 days elapsed. X-irradiated or virus-inoculated leukemic mice normally die 30–40 days after the first detection of thymic lymphomas by palpation. Mice that were inoculated intrathymically with an average of one TCLB cell and that died 20 days later with a thymic tumor of 3 g had experienced ≈32 cell doublings. This would indicate an average maximum doubling time of 16 hr. However, normal cells in the thymus are reported to have a high rate of proliferation and attrition (9), suggesting that injected TCLB cells proliferate in the thymus at an even higher rate.

Cell Cloning in Methyl Cellulose. Cells were plated in 5-cm dishes in 0.8% methyl cellulose in Dulbecco modified Eagle's medium (DME medium) on top of a bottom layer of 1.1% agar in DME medium/10% fetal bovine serum. Routinely, 10^4 or 2×10^4 viable cells were plated per 5-cm dish.

Karyotype Analysis. Cells were incubated for 2 hr at 37°C in medium containing 0.025 μg of colcemid per ml. They were then centrifuged at $150 \times g$ in a clinical centrifuge for 6 min. After aspiration of the medium, the pelleted cells were gently resuspended in 5 ml of 0.75 M KCl for 30 min during which time the cell suspension was mixed continually. Five milliliters of freshly prepared fixative (methyl alcohol/glacial acetic acid, 2:1) was added to the cell suspension. The suspension was allowed to stand for 5 min at room temperature. After two changes of fixative, the cells were placed on glass slides for analysis. G-banding was analyzed after brief exposure to 0.5% trypsin and subsequent staining with Giemsa.

RESULTS

Thymic Versus Splenic Propagation of TCLB Cells. TCLB lines maintain their genotype when propagated for extended time periods in culture, when cloned in methyl cellulose in the presence of exogenous growth factor, and on splenic propagation *in vivo* (8). Intraperitoneal inoculation of 10^4 TCLB cells (female cell line 18-28-4SPL) into male syngeneic B6 mice induced splenic tumors of donor cell origin. (LD_{50} = 5000 cells for i.p. inoculation in 6-week-old B6 mice.) Upon return of these cells to culture no differences were detected between the inoculated cells and the cells that had been passaged through the spleen. The cells were growth-factor dependent, growth-factor secreting, autostimulating, and eudiploid. The phenotype of the specific line used ($TL\text{-}1^-2^-4^-$, TdT^-, $Lyt\text{-}1^+2^-3^-$, $Thy\text{-}1^+$, $IL\text{-}2^+$) was stable through *in vivo* (splenic) passage (8). Furthermore, expression of a panel of oncogenes by TCLB cells did not change after *in vitro* cloning or *in vivo* splenic propagation (unpublished observations).

In contrast, inoculation of TCLB cells into the thymus gave rise to significant genotypic and phenotypic changes in the cells. Male B6 mice in groups of 10 were inoculated intrathymically with 200, 20, or (on the average) 1 female TCLB cells. ($LD_{50} \approx 10$ cells for intrathymic inoculation into 6-week-old B6 mice.) From 16 to 18 days after inoculation, the mice developed large (2–3 g) thymic tumors that were explanted *in vitro*, grown for five to eight transfers (12–20 days; 18–30 cell doublings), and studied by karyotyping, cloning in methyl cellulose, growth-factor dependency, growth *in vivo*, interleukin 2 production, and cell markers. The genotypic and phenotypic changes that were induced by thymic propagation of TCLB cells are described below.

Cell Cloning in Semisolid Medium of Derived TCL Cells. Fig. 1*A* shows clones in semisolid medium of TCL cells after intrathymic inoculation of 20 TCLB cells. Viable cells (2×10^4) from early mass cultures of thymus-passaged TCLB cells ("derived" TCL cells) were plated in methyl cellulose. As controls, 2×10^4 TCLB cells that had been grown in culture but had not been passaged through the thymus were similarly plated. A variable fraction (0.05–0.5%) of cells that

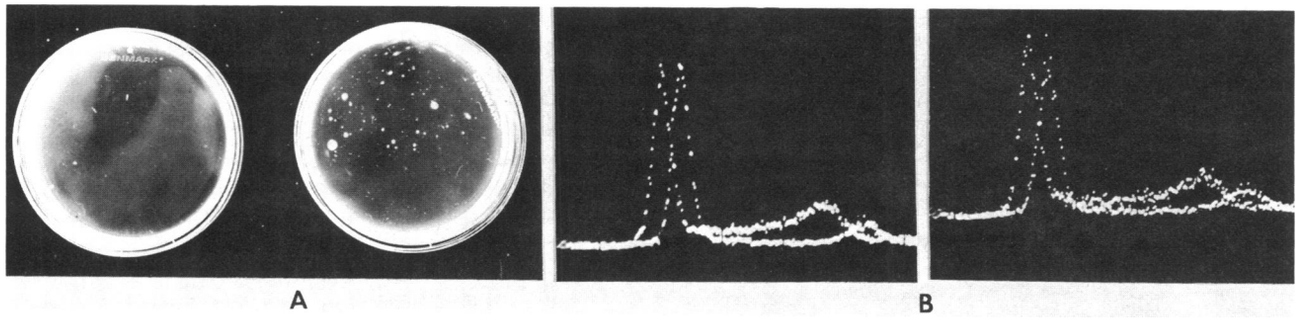

FIG. 1. (*A*) Soft-agar clones of TCL cells that were derived by inoculation of TCLB cells in the thymus. Mouse A3 was inoculated intrathymically with 20 cloned TCLB cells. Nineteen days after inoculation, the resulting thymic tumor was removed and explanted in mass culture. When a suspension lymphoid culture was obtained (at transfer no. 8), 2×10^4 cells were plated in 0.8% methyl cellulose on an agar bottom layer in DME medium containing 10% fetal bovine serum. A control culture in which 2×10^4 donor TCLB cells were similarly plated in semisolid medium is shown on the left. (*B*) Flow microfluorimeter analysis of the DNA content of TCL cultures derived by proliferation of TCLB cells in the thymus. Cells of TCL mass cultures C7 (*Left*) and B1 (*Right*) derived by thymic proliferation of TCLB cells were stained with the DNA-binding dye mithramycin (10, 11) and were analyzed for fluorescence intensity (DNA content) by flow microfluorometry. Eudiploid cloned TCLB cells were analyzed at the same time and their fluorescence distributions were superimposed on the screen and photographed.

had been propagated in the thymus produced colonies in methyl cellulose, while the TCLB cells could not be cloned in semisolid cultures (frequency, $<10^{-6}$).

After thymic propagation of the eudiploid TCLB cells, the average DNA content per cell increased significantly. Thymus-derived TCL cells (10^7) in mass culture were stained with the DNA-binding fluorescent dye mithramycin. The DNA content per cell was estimated by flow microfluorimetry and was compared to the DNA content of similarly stained TCLB cells. Mithramycin fluorescence intensity is proportional to DNA content (10, 11). Cell cycle analysis and fluorescence intensity of the cells in G_1 phase indicated that mass cultures of TCL cells that were derived by intrathymic inoculation of TCLB cells contained 4.5–6% more DNA than the precursor TCLB cells, as is shown in Fig. 1*B*. This indicated that thymus-propagated TCLB cells possessed an increased DNA content per cell as compared to the parental TCLB cells. In control experiments, flow microfluorimetry analysis of the DNA content of TCL cells trisomic for chromosome 15 was compared to that of eudiploid TCLB cells, which showed that the trisomic TCL cells contained an average of 4.9% more DNA than diploid TCLB cells (not shown).

Induction of Chromosomal Abnormalities by Proliferation of TCLB Cells in the Thymus. Table 1 summarizes chromosome counts of TCL cells derived by intrathymic inoculation of TCLB cells into male mice and reisolation of cells from the resulting thymic tumors. After proliferation in the thymus, the explanted mass cultures contained almost exclusively chromosomally abnormal cells, mostly containing 40 (pseudodiploid) or 41 chromosomes (Table 1). In contrast, TCLB cells that were propagated through the spleen (i.p. inoculation, reisolation from the spleen) were genetically and phenotypically stable (Table 1; ref. 8). Most of the cells that were propagated through the thymus and that possessed 40 chromosomes were pseudodiploid and chromosomally quite abnormal, as is shown in Table 2. Deviations from the eudiploid karyotype were mostly attributable to nondisjunction, although translocations have also been observed. The chromosomes that were most often subject to loss or gain were (with declining frequency) chromosomes 14, 15, 16, 3, and 4 (data not shown). Few cells that were trisomic for chromosome 15 were initially encountered among the TCL cells derived by propagation of TCLB cells in the thymus.

An example is the thymic tumor of mouse D1 that was induced in the thymus by injection of (an average of) one TCLB cell (Fig. 2). Of nine single cell clones of the D1 tumor that were grown in methyl cellulose and analyzed, seven contained the translocation in chromosome 4. The source of the translocated segment has not yet been determined. Like other tumors that were derived by injection of TCLB cells into the thymus, the TCL mass culture D1 also displayed considerable chromosome instability over and above the stable translocation in chromosome 4 (Table 2; Fig. 2). Chromosome instability and the induction of aneuploidy and translocations have been observed after inoculation of two other independently isolated TCLB cell lines into the thymus of mice (not shown).

Once explanted and cloned, the derived TCL cells continued to show chromosomal instability in culture. Recloning of

Table 1. Chromosome counts of TCLB cells after their transplantation in the thymus

	Number of cells with *n* chromosomes per cell					
Thymoma culture	39	40	41	42	43	Polyploid
A1		9	1			
A2		7	3			
A2s	1	8				
A3		1	8			
A6		10	1			
A7		6	6			
B1		9	1			
B2	1	8	1			
B3		1	10			
B6		6	4			
B7	1	8	1			
D1-1 through D1-10	2	37	29	7	2	10
E1		3	1			
TCLB-4SPL	1	16				

Cells were explanted *in vitro* and grown for five to eight transfers in mass culture. Cloned eudiploid female TCLB cells (cell line 4SPL) were inoculated intrathymically into male syngeneic B6 mice. The number of cells injected per thymus was 20 (A), 200 (B), or 1 (D and E). Chromosome counts of the donor cells before intrathymic inoculation are shown on the bottom line. One mouse, A2, also developed a splenic T-cell neoplasm, due to the spilling of TCLB cells during injection of the cells into the thymus. Cells of the splenic mass culture (A2s) were identical to the donor eudiploid TCLB cells, while cells of the thymic tumor were aneuploid or pseudodiploid.

Table 2. Some examples of karyotypes of TCLB cells after transplantation in the thymus

Culture	No. of chromosomes	Karyotype
A6	40	3^+ 5^- 9^{++} 14^- 18^- 19^+
	41	4^- 6^+ 14^+
A7	40	Normal
A8	40	5^- 9^+
B1	41	7^- 14^+ 16^+
B3	41	3^- 8^{++} 11^{++} 14^- 16^- 17^+ 18^- x^+
B6	41	3^+ 11^+ 15^-
	40	4^- 13^+ 14^- x^+
C3	41	t(4) 6^+ 7^- 15^+
	41	t(4) 6^+ 8^+ 16^-
D1-1	41	t(4) 6^+ 10^+ 11^- 12^- 14^+
D1-9	43	t(4) 7^+ 9^+ 12^- 15^+ 17^+
	42	t(4) 8^- 13^+ 16^+ 17^+ 18^- x^+
TCLB-4SPL	40	Normal diploid

Cloned, normal diploid female TCLB cells (cell line 4SPL) were inoculated into the thymus of male syngeneic B6 mice. The number of cells injected per thymus was 20 (A), 200 (B), or 1 (D and E). Chromosome counts of the donor cells before intrathymic inoculation are shown on the bottom line. Translocation of the chromosomal segment of unknown origin to chromosome 4 has been observed (see Fig. 2). Data for cell clones D1-1 and D1-9 refer to cells that were twice cloned in methyl cellulose, then grown in mass culture, and prepared for karyotyping. All cells were of donor (female) origin.

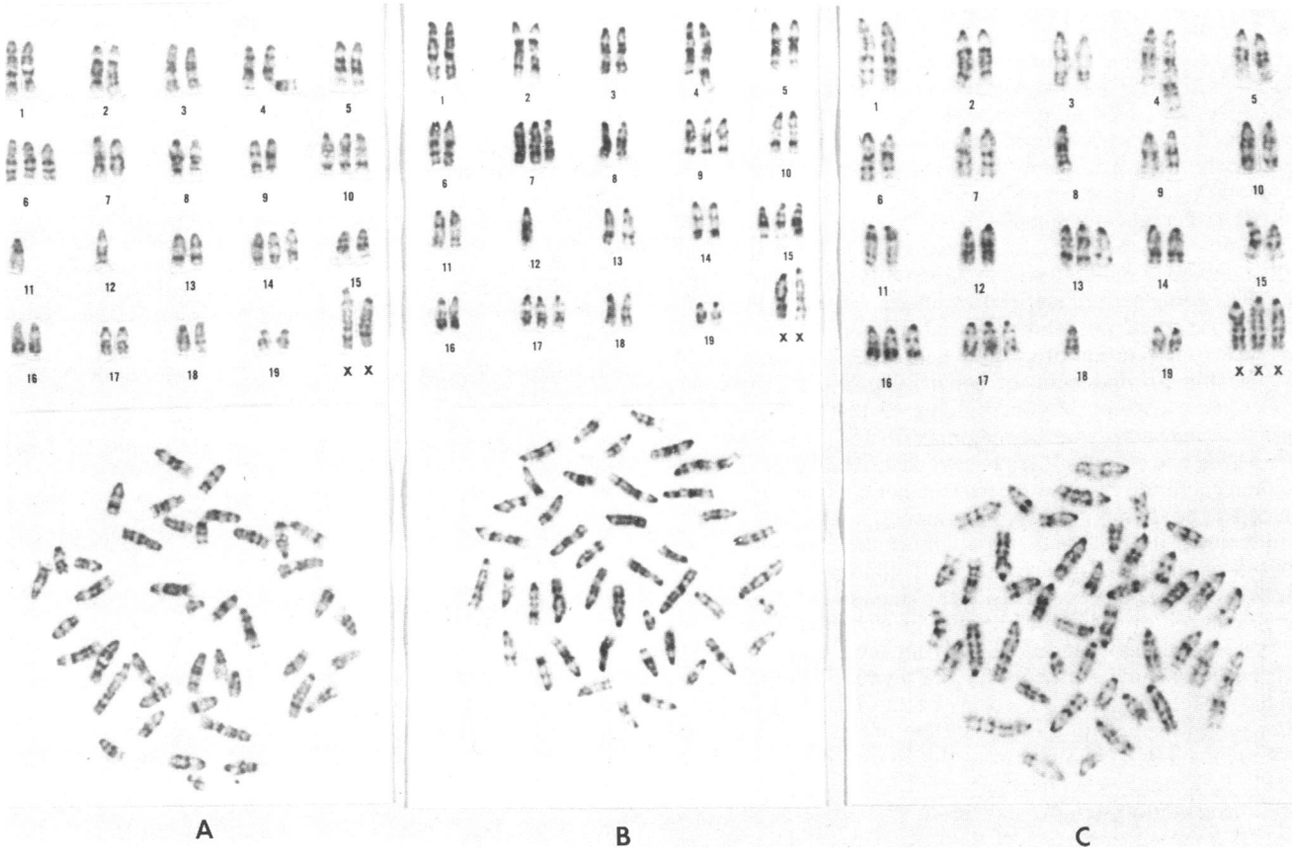

FIG. 2. Karyotypes of thymus-induced TCL cells of clones D1-1 (*A*) and D1-9 (*B* and *C*). Clones in methyl cellulose were grown from the thymus-induced TCL mass culture D1 (derived by intrathymic inoculation of an average of a single TCLB cell). Cells of clones D1-1 through D1-10 had unstable karyotypes. Karyotypes of one cell of clone D1-1 and two cells of clone D1-9 are shown. These possessed the karyotypes indicated in Table 2.

the cells in soft agar revealed new aneuploid or pseudodiploid segregants from a single clone. This was also the case for the D1 tumor cells when passaged in culture (Fig. 2; Table 2). The instability of the derived TCL cells is notable in comparison to the phenotypic and genotypic stability *in vivo* and *in vitro* of the eudiploid TCLB cells. It also contrasts with the stability of the TCL cells trisomic for chromosome 15, which were isolated from frank thymic lymphomas after a 3–5 month period of latency (8).

Tumorigenic Properties of Derived TCL Cells. After proliferation of TCLB cells in the thymus, derived TCL cells produced tumors at the site of subcutaneous injection. Mass cultures were established from thymic tumors induced by intrathymic inoculation of (an average of) one TCLB cell. The resulting TCL cells were cloned in methyl cellulose and 10 clones were picked at random and grown. Cells (10^4) of clones D1-1 to D1-10 were injected subcutaneously into groups of 6-week-old male mice. The cloned derived TCL cells gave rise to walnut-sized subcutaneous tumors at the site of injection in 100% of injected mice in 2 weeks. In control groups, no local tumors developed in mice similarly injected with 10^4 TCLB cells, although 9 out of 10 of the control mice developed splenic tumors in 5–6 weeks.

DISCUSSION

We have previously shown (8) that x-irradiated mice or mice that had been inoculated with RadLV often harbor two stable distinct T-cell neoplasms, one composed of growth-factor-dependent eudiploid TCLB cells that did not grow under the skin of mice or clone in semisolid medium, and a thymic neoplasm composed of growth-factor-independent, monoclonal, trisomic TCL cells, which generated local subcutaneous tumors at the site of inoculation and could be quantitatively cloned in semisolid medium. We have designated the corresponding tumors T-cell neoplasms of phase I (lymphoblastomas), and phase III (lymphomas), respectively. The cells of phase I neoplasms and the cells of phase III neoplasms bred true through growth *in vitro* and *in vivo* as long as phase I cells were not propagated through the thymus.

Inoculation into the thymus of one or a few cloned TCLB cells generated a family of donor-derived aneuploid or pseudodiploid neoplastic T cells. Among 120 cells that have been karyotyped, 117 cells were of female donor origin, while 3 cells had lost one X chromosome. None of the cells studied had a Y chromosome and was of recipient (male) origin. Unlike the donor TCLB cells (phase I) or the often-described (phase III) TCL cells that are trisomic for chromosome 15 (12–14), the thymic tumors that were derived by inoculation of TCLB cells were genotypically highly unstable. They had properties intermediate between the stable phases I and III T-cell neoplasms. Therefore, we will refer to the thymic neoplastic cells that were derived by inoculation of TCLB cells into the thymus as phase II TCL neoplastic cells.

Phase II TCL cells were chromosomally highly unstable. Even within single cell clones grown in semisolid medium, many different chromosomal abnormalities were found. Recloning gave rise to an additional spread of aneuploid cells. It is not yet clear whether chromosomally stable TCL cells can be isolated *in vitro* from a population of genetically unstable, evolving, phase II TCL cells, or how many cycles of single-cell cloning are required to select for putative genetically stable TCL clones. Nor is it clear what the genotype(s) of such stable clones will be.

Analysis of the karyotypes of phase II TCL cells indicates

that mostly they resulted via nondisjunction. Proliferation of TCLB cells in the thymus often resulted in the induction of stable chromosome translocations (Fig. 2), which were superimposed on a continuously evolving aneuploidy. We propose that the induction of chromosome abnormalities that is associated with the proliferation of growth-factor-dependent T blasts in the thymus plays a central role in the generation of growth-factor-independent monoclonal TCL cells. Through selection among many possible aneuploid and pseudodiploid cells, growth-factor-independent genetically stable cells may grow and give rise to apparently monoclonal thymic TCLs. Furthermore, specific mutations that are generated as a result of this chromosomal instability may be required for the fixation of a stable genotype. The generation of growth-factor-independent genetically stable TCL cells possessing a trisomy of chromosome 15 that has been observed in murine thymic lymphomas (8, 15–17) may therefore be the end result of the process described here—namely, the generation of organ-specific genetic havoc that is induced by the thymic microenvironment.

In contrast to the genetic instability of the experimentally derived phase II TCL cells, thymic lymphomas that are induced by x-irradiation, virus-inoculation, or by chemicals have been reported to be composed of stable trisomic cells (12–19); the class of chromosomally unstable phase II TCL cells has not been described. Phase II thymic TCL cells may not have been observed previously because a cell transplantation step was used in the preparation of the TCL cells for karyotyping (14–17). Such transplantation selects for the growth-factor-independent trisomic TCL cells. By avoiding the transplantation step, we have found that many (>60%) x-ray-induced and virus-induced thymic TCL cells are composed of genetically unstable, phase II lymphoma cells. Neoplastic T cells of phase II are often found in thymic lymphomas of x-irradiated and virus-inoculated mice, and thus are not an experimental artefact (unpublished results).

It is not known how the thymic microenvironment induces chromosomal abnormalities in proliferating TCLB cells. One can envisage a positively acting mechanism (e.g., by means of a thymus-specific growth factor) or a negatively acting mechanism: forced proliferation in the absence of growth factor(s) may induce unbalanced growth and chromosome abnormalities. In addition, the replication of mink-cell focus-inducing thymotropic virus may contribute to the observed genetic havoc. Even if the first aberrant mitoses are triggered by the thymic microenvironment, it is striking that they continue when the cells are removed from it. What is imposed or derepressed in the thymus is not a transient response to environmental conditions but rather a long-term genetic instability. The mechanism of thymus-induced chromosome abnormalities should be experimentally testable now that TCLB cells that are susceptible to the thymic microenvironment are available *in vitro*.

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