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Induction of multiple independent T-cell lymphomas in rats inoculated with Moloney murine leukemia virus

(oncogenesis/thymoma/tumor clonality)

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ABSTRACT **Tumor cell DNA derived from different lymphoid organs of 30 rats serially inoculated at birth with Moloney murine leukemia virus (MoMuLV) was examined by Southern blot analysis and hybridization to the following DNA probes: MoMuLV long terminal repeat (LTR), Moloney leukemia virus integration regions 1, 2, 3, and 4 (*Mlvi*-1, *Mlvi*-2, *Mlvi*-3, and *Mlvi*-4), T-cell receptor β locus, and immunoglobulin heavy chain locus. This analysis revealed that the tumors segregating in different lymphoid organs in 10% of the animals were clonally unrelated. These findings are consistent with the hypothesis that the MoMuLV-induced rat thymic lymphomas are polyclonal in origin. At least two factors may be responsible for this phenomenon: (*i*) increase in the number of the available target cells in virus-infected animals, and (*ii*) genetic instability associated with provirus integration in the developing premalignant clones.**

Oncogenic retroviruses that lack a transforming gene are responsible for several types of neoplasms in infected animals (for review, see ref. 1). Common features shared by these neoplasms are that they are clonal and they develop after a long latency period (1). The latency period of T-cell lymphomas induced in mice and rats by Moloney murine leukemia virus (MoMuLV), a representative of this class of viruses, ranges between 2 and 6 months (1). The clonal nature and the long incubation period of tumor induction have been interpreted to suggest that tumor induction is the result of a single rare event (1–3). The monoclonal origin of these tumors, however, has been challenged by the finding that multiple preleukemic cells—i.e., partially transformed cells capable of developing the tumor cell phenotype—appear very soon after virus inoculation (4). The selection of a single tumor cell population out of the pool of multiple preleukemic cell clones could therefore depend on multiple, sequential genetic changes and not on a single rare genetic event (5, 6). To test the hypothesis that MoMuLV-induced thymic lymphomas were polyclonal in origin, we examined the clonal relationship of tumors derived from different lymphoid organs of the same animal in a group of 30 rats inoculated serially with MoMuLV. We reasoned that if the induced tumors originated from a polyclonal pool of preleukemic cells, then clones of transformed cells derived from independent transformation events could have segregated in different lymphoid organs in the early stages of oncogenesis. This segregation could allow for the independent evolution of two clonally unrelated neoplasms in a single animal. Our analysis revealed that 10% of the tumor-bearing animals carry two clonally unrelated and independently segregating neoplasms.

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MATERIALS AND METHODS

Tumors. Long-Evans rats were inoculated intraperitoneally (ip) with 5×10^5 XC plaque-forming units (7) of wild-type MoMuLV within 24 hr after birth. Two to 6 months after virus inoculation all the animals developed thymic lymphomas. A complete autopsy was performed on all the sacrificed rats and tissues from multiple-tumor-infiltrated organs were snap-frozen and kept at −70°C.

Isolation of Genomic DNA and Southern Blotting. Isolation of high molecular weight genomic DNA was carried out using standard procedures as previously described (5). Southern blot analysis of genomic DNA and hybridization to multiple DNA probes was also carried out according to standard procedures (5). When the probe derived from the murine T-cell receptor β locus was used, the hybridization was carried out at low stringency in 40% (vol/vol) formamide and 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C. The filters hybridized under low stringency conditions to the murine T-cell receptor β probe were washed three times with 2× SSC/0.1% $NaDodSO_4$ at room temperature and once with 1× SSC/0.1% $NaDodSO_4$ at 65°C for 30 min.

DNA Probes. The following DNA probes were used: MoMuLV long terminal repeat (LTR) (8), pPVa (*Mlvi*-1) (5, 8), pTS6 (*Mlvi*-2) (5), pM6 (*Mlvi*-3) (9), pLE18 (*Mlvi*-4) (unpublished), pJβ2b (murine T-cell receptor β) (ref. 10, Fig. 3*B*), and pRI18 (rat *Igh*) (11). The murine T-cell receptor β probe was kindly provided by Walter Schuller (Fox Chase Cancer Center). All the other probes originated in this laboratory.

RESULTS

Genomic DNA from tumor tissue derived from multiple organs of 30 serially inoculated tumor-bearing animals was digested with *Eco*RI, which does not cleave the MoMuLV genome (12). After agarose gel electrophoresis and transfer to nylon membranes it was hybridized to a MoMuLV LTR probe. This analysis revealed that there was no similarity in the pattern of provirus integration between the tumors derived from two different organ sites of 3 out of 30 animals (Fig. 1). This was in sharp contrast to the remaining 27 rats, represented by rat 6892 in Fig. 1, in which the tumors isolated from different lymphoid organs showed a related pattern of provirus integration. Since tumor induction is linked to provirus integration in the vicinity of cellular protooncogenes (for review, see ref. 1), we interpreted this finding to suggest that 10% (3/30) of the tumor-bearing animals carried at least two neoplasms, which were derived from two independent transformation events.

To confirm these data we examined the clonal relationship between the tumors infiltrating different lymphoid organs of

Abbreviations: MoMuLV, Moloney murine leukemia virus; LTR, long terminal repeat.

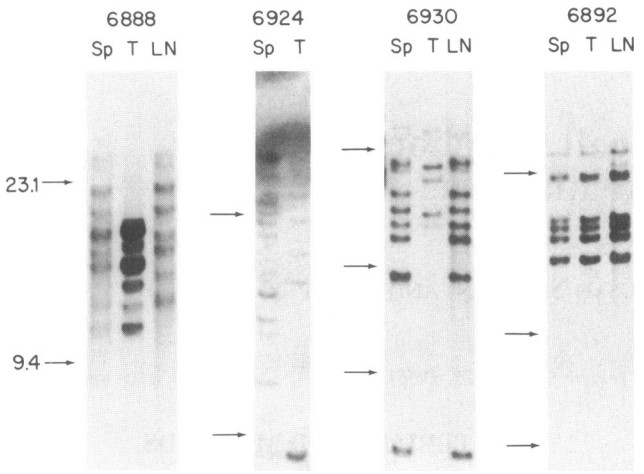

FIG. 1. Southern blot analysis of *Eco*RI-digested tumor cell DNA isolated from multiple lymphoid organs of four independent tumor-bearing animals. Hybridization was performed with a MoMuLV LTR probe. Sp, T, and LN refer to spleen, thymus, and lymph nodes, respectively. The arrows indicate the position of the 23.1-, 9.4-, and 6.5-kilobase (kb) *Hin*dIII phage λ fragments.

the same animals by using three additional clonal markers: (*i*) provirus insertion in a set of loci of common integration (*Mlvi*-1, *Mlvi*-2, *Mlvi*-3, and *Mlvi*-4), (*ii*) rearrangement of the T-cell receptor β locus, and (*iii*) rearrangement of the immunoglobulin heavy chain (*Igh*) locus.

Two out of three animals carried tumors that contained a provirus in the *Mlvi*-1 locus (Fig. 2). However, this genetic change was detected only in the tumor derived from a single organ site in each animal. That is, in animal 6924 only the tumor derived from the spleen contained a provirus in *Mlvi*-1, while in animal 6930 a provirus in *Mlvi*-1 was present only in the tumor isolated from the thymus. The remaining loci of common integration were not rearranged in these tumor tissues.

To analyze the genomic DNA of the same tumors for rearrangements in the T-cell receptor β locus, we generated a restriction map of this locus in the rat by digesting genomic rat DNA with several restriction endonucleases and combinations of them. The blots were hybridized to the pJβ2b probe, which is derived from the Jβ2 region of the murine T-cell receptor β locus (Fig. 3*B*). On the basis of this map we analyzed the tumor cell DNA for rearrangements in this locus by digestion with *Sac* I and hybridization to the pJβ2b probe.

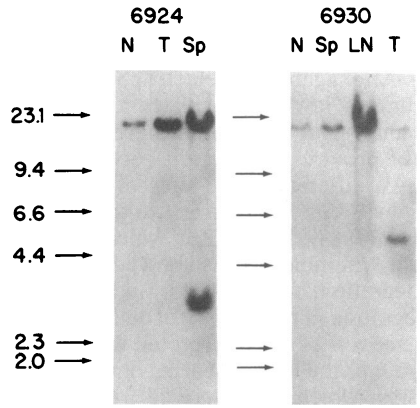

FIG. 2. Southern blot analysis of *Bam*HI-digested normal rat and tumor DNA isolated from multiple lymphoid organs of two independent tumor-bearing animals. Hybridization was performed with the *Mlvi*-1 probe pPVa. N, Sp, LN, and T refer to normal, spleen, lymph node, and thymus, respectively. The arrows indicate the position of the fragments generated by *Hin*dIII digestion of phage λ DNA.

This generated a 20-kb germ-line restriction fragment which included both the β1 and β2 loci (Fig. 3*B*). The results (Fig. 3*A*) revealed rearrangments of the T-cell receptor β locus in all the tumor tissues examined, thus confirming their T-cell origin. The rearrangements detected in tumor tissues infiltrating different lymphoid organs, however, were unrelated, indicating again that all these animals carried at least two clonally independent tumors.

Rearrangements in the immunoglobulin heavy chain locus (*Igh*) are frequently detected in hematopoietic tumors of non-B-cell origin (13–15). In the case of T-cell lymphomas induced in rats by MoMuLV, rearrangements in the *Igh* locus were detected in approximately 60% of the tumors (unpublished). On this basis, the tumors isolated from the animals 6888, 6924, and 6930 were analyzed for rearrangements in the *Igh* locus (Fig. 4). On the basis of the restriction map of this locus in the rat (11), tumor cell DNA was digested with *Eco*RI and hybridized to the pRI18 probe derived from the constant region of the rat *Igh* locus. The tumors derived from two of the animals (6924 and 6930) showed rearrangements of this locus. On the basis of these rearrangements the tumor infiltrating the spleen and the lymph nodes of one of these animals (6930) was distinguished from the tumor infiltrating the thymus, suggesting the clonal independence of these tumors.

DISCUSSION

The data presented in this manuscript showed that at least two independent lymphomas can segregate in two different lymphoid organs of a single animal. This was demonstrated by Southern blot analysis of tumor cell DNA and hybridization to the MoMuLV LTR, an *Mlvi*-1, a T-cell receptor β, and an *Igh* probe. This analysis revealed that the tumors developing in different lymphoid organs of 10% of the animals did not share any clonal markers, indicating that they were derived from independent transformation events (16). This is not surprising if we consider the events preceding tumor induction by MoMuLV. Within weeks after inoculation of mice or rats with this virus, a polyclonal proliferation of hematopoietic precursor cells is detected in the bone marrow and spleen of the inoculated animals (17–21). Furthermore, adoptive transfer experiments have shown that the spleen and the bone marrow of these animals contain multiple clones of preleukemic cells—i.e., partially transformed cells capable of assuming the tumor cell phenotype (4, 22, 23).

A likely explanation for these results is that the virus recognizes a large number of target cells which upon infection enter the preleukemic cell pool. In addition, since the provirus can efficiently reintegrate in the genome of infected preleukemic cells (unpublished data), the continuous presence of the virus is responsible for a high degree of genetic instability which increases the likelihood that any one of these cells will become fully transformed. This scenario would predict the development of multiple tumors in most animals. Indeed, the number of clones that become fully transformed may be underestimated by our data. The detection of only two independent tumors in a single animal may be a low estimate of the number of independent transformation events, because convergent evolution of tumor cell clones occurring in the final stages of oncogenesis favors a few rapidly growing clones of tumor cells (24). Furthermore, the detection of multiple tumors in only 10% of the rats represents a minimal estimate of the number of animals carrying more than one independent thymoma. This is because the design of the study allowed the detection of independent tumors only if they segregated in different organs. Our previous studies, however, have suggested that the thymus of a single tumor-bearing animal may contain multiple independent tumor cell clones (5).

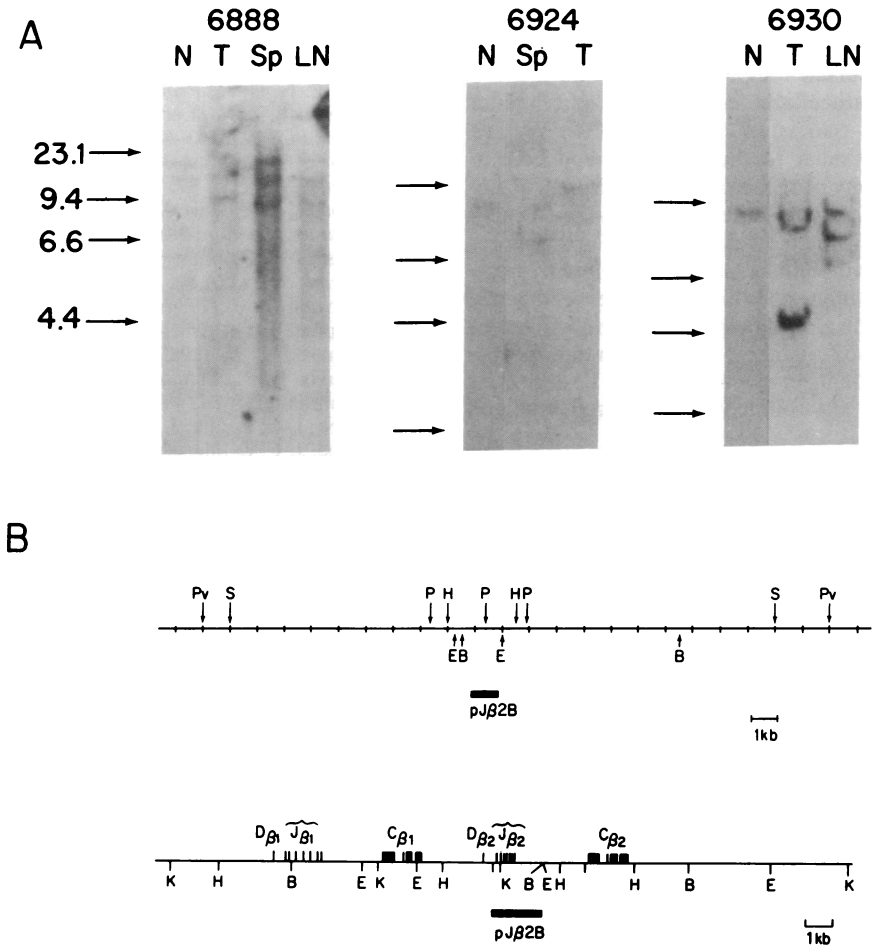

FIG. 3. (*A*) Southern blot analysis of *Sac* I-digested normal rat and tumor DNA isolated from multiple lymphoid organs of three independent tumor-bearing animals. Hybridization was performed with the pJβ2b probe derived from the murine T-cell receptor β_2 locus. (*B*) The lower map is of the murine T-cell receptor β locus, indicating the origin of the probe. The upper map is a restriction map of the rat T-cell receptor β locus. This map was generated by hybridization of the pJβ2b probe to normal rat DNA, digested with multiple restriction endonucleases. Restriction endonucleases: Pv, *Pvu* II; S, *Sac* I; P, *Pst* I; H, *Hin*dIII; K, *Kpn* I; B, *Bam*HI; E, *Eco*RI.

If this hypothesis is correct, we would expect that multiple primary tumors will be detected in a variety of situations which enhance the number of the available target cells and/or promote genetic instability in the developing premalignant cell clones. This has been observed in several hereditary defects which predispose to the development of neoplasia. These include conditions which are characterized by mutations in a set of tumor suppressor genes whose homozygous inactivation is responsible for tumor induction (25). This type of genetic defect is probably responsible for a dramatic increase in the number of the available target cells. Another type of genetic defect which also leads to the development of multiple independent tumors is observed in the rare autosomal recessive disorders xeroderma pigmentosum (26), ataxia–telangiectasia (27), Bloom syndrome (28), Fanconi anemia (28), and possibly multiple endocrine neoplasia type II (29). These disorders are associated with genetic instability in the somatic tissues of the affected individuals. This would increase both the number of target cells and the frequency of secondary genetic events in the developing premalignant cell clones.

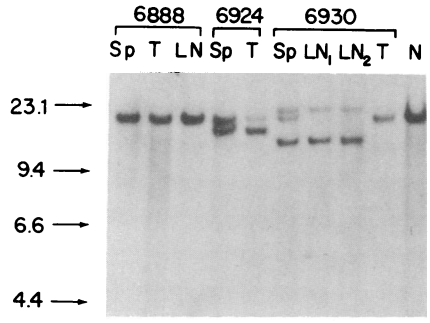

FIG. 4. Southern blot analysis of *Eco*RI-digested normal (N) and tumor DNA isolated from multiple lymphoid organs of three independent animals. Hybridization was performed with the pRI18 *Igh* probe.

These results may be useful in forming predictions regarding the etiology of human neoplasms. The detection of multiple, clonally unrelated, nonhereditary tumors in a single individual can be interpreted to suggest that the transforming agent initiates at least partial transformation in multiple clones of target cells, and it is responsible for genetic instability in these partially transformed cells. Since there is no clear evidence that partially or fully transformed cells have an intrinsically high degree of genetic instability (30–32), this property may depend on the continuous presence of the transforming agent throughout the oncogenic process. Two types of insults may be responsible for such an effect: continuous long-term environmental exposure to chemical carcinogens or ionizing irradiation, and exposure to oncogenic viruses. Currently, viral etiology has been implicated in only a small percentage of human tumors. These include adult T-cell leukemia (ATL) which is associated with infection with the human T-cell lymphotropic virus type I (HTLV-

I), and a variety of other T-cell neoplasms which are occasionally associated with HTLV-I infection (33); Epstein–Barr virus (EBV)-induced Burkitt lymphomas; hepatitis B virus (HBV)-associated hepatocellular carcinomas; and human papilloma virus (HPV)-associated carcinomas (34). We are not aware of a study similar to the one described here in patients infected with any of these oncogenic viruses. However, it is known that individuals infected with HTLV-I carry multiple clones of virus-infected cells (35, 36). Furthermore, EBV-induced Burkitt lymphomas may recur following treatment, and the clone of tumor cells emerging after recurrence may be different from the clone composing the original tumor (37, 38). Analsysis of the clonal nature of human tumors may therefore be helpful in identifying those that may be due to the long-term exposure to environmental carcinogens such as oncogenic viruses.

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