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Chemically induced murine T lymphomas: Continued rearrangement within the T-cell receptor β-chain gene during serial passage

(3-methylcholanthrene/N-methyl-N-nitrosourea)

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ABSTRACT **The first constant region of the *Tcrb* gene was completely deleted from the DNA of 8/10 mouse cell lines established from 3-methylcholanthrene-induced RF/J thymic lymphomas, but 6/7 primary lymphomas contained the first constant region sequences. DNA from RF/J thymic lymphomas induced by *N*-methyl-*N*-nitrosourea was then examined serially as the tumors were passaged *in vivo* and adapted to growth in culture as uncloned and, in some cases, cloned lines. Patterns of *Tcrb*-specific restriction fragments from most tumors changed extensively during continued propagation. Analysis of the patterns often suggested that initial DNA rearrangements within the *Tcrb* complexes of monoclonal tumors had been followed by further rearrangements within the same genes. However, these different patterns may alternatively have represented successive outgrowth of separate lineages from lymphomas that were polyclonal in origin.**

About 25% of mice of the inbred RF/J strain develop spontaneous thymic lymphoma starting at 10 mo of age and continuing into their second year. These mice are also exquisitely sensitive to certain lymphomagens: treatment at early ages with the carcinogens 3-methylcholanthrene (MeCA) or *N*-methyl-*N*-nitrosourea (MeNU) induces a near 100% incidence of thymic lymphoma before 10 mo of age (1–3). Because these tumors consist of cells of the T-lymphocyte lineage (4), we chose to characterize these cells with respect to the composition of the genes governing their T-cell receptor (TCR) molecules. Somatic rearrangement of DNA within these genes must occur during T-cell differentiation to permit expression of mature TCR structures (5).

We now report our findings concerning the patterns of DNA rearrangement within the *Tcrb* gene (chromosome 6) that governs the β subunit of the complex TCR structure (6). We determined the patterns of *Tcrb* gene-specific DNA restriction fragments from chemically induced RF/J thymic lymphomas studied as primary tumors, tumors passaged *in vivo*, lines of tumor cells adapted to growth in culture and, in some cases, clonal derivatives of these cell lines. Our results indicate that patterns of *Tcrb* gene rearrangement in a given lymphoma frequently show marked differences with continued propagation of the cells *in vivo* or in culture. In some cases these changes appear consistent with the interpretation of successive rearrangements within already rearranged *Tcrb* segments. However, in most cases the alternative interpretation of a polyclonal origin of the tumors cannot be ruled out.

MATERIALS AND METHODS

Chemically Induced Thymic Lymphomas. Treatment of female mice of the RF/J strain, obtained from The Jackson Laboratory, with either MeCA or MeNU induced high incidences of thymic lymphoma. In one treatment, a 1% (wt/vol) solution of MeCA in benzene was applied with a brush daily for 5 days to the shaved backs and flanks of 3-mo-old mice; lymphomas were detected in most mice 2–5 mo later. In another treatment, 5- or 9-week-old mice received five weekly i.p. injections of 0.35 ml of a solution of MeNU at 2 mg/ml in standard saline citrate (0.15 M sodium chloride/0.015 M sodium citrate)—each dose corresponding to ≈30 mg/kg of body weight; all mice in both age groups developed thymic tumors 8–14 weeks after the first injection. The occurrence of tumors was detected mainly by the labored breathing of the hosts.

Thymic lymphomas were passaged s.c. in syngeneic mice. After four to seven serial passages, the tumor cells were seeded at a density of 10^6 cells per ml in Dulbecco's minimal essential medium (DMEM) with 20% heat-inactivated fetal calf serum containing 50 μM 2-mercaptoethanol and incubated at 37°C in 5% CO_2/95% air. After establishment of stable suspension lines, 2-mercaptoethanol concentrations were gradually reduced to zero. Cells of MeNU-induced lymphoma lines were cloned in low-melting agarose using RPMI 1640 medium containing 20% fetal calf serum and 50 μM 2-mercaptoethanol and mixed with an equal volume of conditioned medium. Colonies were picked after 1 week, grown in conditioned RPMI 1640 medium for another 2 days, and then passed in DMEM with 20% fetal calf serum and 50 μM 2-mercaptoethanol; 2-mercaptoethanol was later withdrawn from the medium.

DNA Extraction and Hybridization. Cells from tumors and cultured lines were lysed with $NaDodSO_4$ and treated with proteinase K. After a 4-hr incubation, DNA was extracted with phenol and chloroform and finally dialyzed against TE buffer (10 mM Tris/1 mM EDTA). After digestion with restriction enzyme *Hin*dIII, DNA fragments were separated by electrophoresis through 0.8% agarose at 24 mA of constant current for 24 hr and transferred to nitrocellulose (7).

Hybridization Probes. DNA probes recognizing portions of the *Tcrb* or *Igh-1* (immunoglobulin heavy chain) genes were hybridized to nitrocellulose blots of tumor cell DNA. RBL5-17 is a cDNA clone containing about half the coding region plus 3′ untranslated sequences of the first constant region (*C1*) segment of the *Tcrb* gene (8); it is an 800-base pair *Pst* I fragment cloned in pBR322. Among *Hin*dIII fragments of RF/J germline DNA, this probe hybridizes with a 9.5-kilobase (kb) fragment that includes the *D1*, *J1*, and most of the *C1* coding regions, where D represents diversity region, J represents joining region, and C represents constant region of TCR β chain. Jβ2A is a genomic clone consisting of the

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Abbreviations: TCR, T-cell receptor; MeCA, 3-methylcholanthrene; MeNU, *N*-methyl-*N*-nitrosourea; *V, C, J,* and *D*, genes for variable region (V), constant region (C), joining region (J), and diversity region (D) of TCR β chain.

*Hin*dIII–*Bam*HI fragment of *Tcrb* encompassing *D2.1* and the entire *J2* region (9). This fragment hybridizes with a 5.2-kb *Hin*dIII fragment of RF/J germ-line DNA that includes the *D2* and *J2* coding regions (5). Both probes were provided by T. W. Mak (Ontario Cancer Institute, Toronto). The J11 probe includes the *J3* and *J4* regions of the *Igh-1* gene and was provided by T. V. Rajan (Albert Einstein College of Medicine). The RBL5 and Jβ2A probes were isolated from their plasmids after restriction endonuclease digestion and electrophoretic separation. Probe J11 was used intact without isolating the insert. These probes were radiolabeled by nick-translation. Hybridization and washing were done as described by Herr and Gilbert (10).

RESULTS

Experimental Design. The β subunit produced by the *Tcrb* gene is a glycoprotein molecule comprising an amino-terminal variable domain and a constant domain anchored in the cell membrane by a carboxyl-terminal hydrophobic region. The variable domain is the product of a 5′ portion of the gene constituted during T-cell differentiation by somatic rearrangement of multiple germ-line DNA segments. These DNA rearrangements result in the juxtaposition of previously separate coding segments from the *V* (variable), *D*, and *J* subregions of the gene. In late-stage T-cells, this rearranged 5′ variable (*V–D–J*) portion of the gene and a 3′ portion encoding a constant domain are expressed as a single RNA species (11). The *Tcrb* gene complex on mouse chromosome 6 (6, 8) includes multiple *V* region sequences in its 5′ portion and two sets of *D*, *J*, and *C* segments in its 3′ portion. Thus, the order of potential coding segments in the germ-line configuration of this gene is: $(V_1–V_n)–D1–(J1_1–J1_n)–C1–D2–(J2_1–J2_n)–C2$ (Fig. 1). *Tcrb* gene products expressed by mature T lymphocytes generally arise from DNA rearrangements comprising a *V* segment plus either a *D1–J1–C1*, a *D1–J1–C2*, or a *D2–J2–C2* combination.

Interpretation of our data is based on the following assumptions. The first step in the process of *Tcrb* gene DNA rearrangement involves looping out of DNA between *D* and *J* segments to form *D1–J1* and/or *D2–J2* juxtapositions on each chromosome 6. In cells possessing such rearrangements, *Hin*dIII fragments hybridizing with the RBL5 and Jβ2A probes are smaller than their respective 9.5- and 5.2-kb homologues in germ-line DNA. Next, the DNA between a *V* segment and a *D–J* joining is looped out to form a *V–D1–J1* or *V–D2–J2* juxtaposition. When a *V–D1–J1* joining is formed, the resulting *Hin*dIII fragment detected by the RBL5 probe will usually be larger than its germ-line homologue, and the Jβ2A probe will detect a fragment of germ-line size or smaller. When a *V–D2–J2* joining is formed, the looped-out DNA between the *V* and *D2–J2* segments will include the entire set of sequences hybridizing with the RBL5 probe, and thus no RBL5-specific fragment will be identified; in this case, the Jβ2A probe will usually identify a fragment larger than its germ-line homologue. Although only the *Tcrb* gene on a single chromosome 6, if any, will achieve a productive rearrangement in a particular developing T lymphocyte, its homologue on the second chromosome 6 will usually also undergo some degree of rearrangement or deletion.

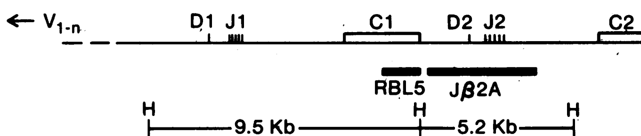

FIG. 1. Map of the portion of the germ-line *Tcrb* gene encoding the two sets of *D*, *J*, and *C* regions of the gene product. Three *Hin*dIII sites (H) are indicated together with the sizes of the *Hin*dIII restriction fragments hybridizing with the RBL5 and Jβ2A probes, respectively.

MeCA-Induced Lymphomas. Initial studies of the state of the *Tcrb* gene were done using cells from MeCA-induced RF/J lymphoma lines previously established in culture in this laboratory (4). Frozen stocks from relatively early passages of ten lines were reestablished in culture, and DNA was extracted from them and cut with *Hin*dIII. In eight of the ten lines, the RBL5 probe failed to hybridize detectably with any restriction fragment, indicating that the *C1* region of the gene had been completely deleted from each chromosome 6. However, the Jβ2A probe did hybridize with one or more DNA fragments in each tumor, suggesting that the *Tcrb* genes have been rearranged for potential use of their *D2–J2–C2* segments (data not shown). These findings agree with those from studies of established lines of T-lymphoma cells in other laboratories, although only a small minority of lines established from normal helper or cytotoxic T cells showed complete deletion of the *C1* region (12).

To determine whether loss of the *C1* region of the gene was also a frequent characteristic of primary MeCA-induced lymphoma cells, as well as of cell lines derived from them, new RF/J lymphomas were induced by treatment with MeCA. DNA from only one of seven primary lymphomas studied was devoid of fragments hybridizing with the RBL5 probe. Taken together, these findings suggested that DNA rearrangement within the *Tcrb* gene continues to occur during the numerous cell generations required for establishment of stable culture lines.

MeNU-Induced Lymphomas. To investigate the question of possible sequential DNA rearrangement within the *Tcrb* gene during passage of T-lymphoma cells, we induced a further set of such tumors in RF/J mice by i.p. treatment with MeNU. DNA was extracted from portions of six primary tumors, from selected passages *in vivo* of each tumor, from cells of early passages of uncloned lines established in culture, and, in some cases, from clones derived from established tumor cell lines. Blots of *Hin*dIII fragments were first probed with RBL5 for analysis of rearrangement in the *D1–J1* cluster and later reprobed with the Jβ2A probe for analysis of rearrangement in the *D2–J2* region.

MNU31. Fig. 2*A* shows restriction fragments identified in DNA from derivatives of tumor MNU31 by probes RBL5 (*Upper*) and Jβ2A (*Lower*). With the RBL5 probe, cells from both the primary tumor and the cultured line (lanes P_0 and TC) showed complex patterns with three bands at 11.5, 7.8, and 6.1 kb plus several submolar bands. However, these same samples showed only a single major band at 3.7 kb when probed with Jβ2A. Assuming an immunoglobulin-like looping-out and joining model, the RBL5 bands represent *V–D1–J1* joining, and the Jβ2A band represents *D2–J2* joining. Karyotypic analysis of the MNU31 cell line at passage four showed trisomy of the *Tcrb*-bearing chromosome 6 (unpublished data). Thus, the three major RBL5 bands may represent different *V–D1–J1* rearrangements on each chromosome or, alternatively, they may indicate that, following an initial *D1–J1* joining early in tumor development, different subclones appeared with different *V* segments joined to the original *D1–J1* joining(s).

To further analyze MNU31, clones from the cell line were established and analyzed similarly. Results from four representative clones are included in Fig. 2*A*, lanes b, e, f, and h. Each subclone showed either one or two clear bands with both probes, but three different overall patterns emerged among the clones. All clones showed the same 6.1-kb RBL5 band and the same 3.7-kb Jβ2A band present in the primary tumor, but each clone showed one or two bands that had been absent or very weak in the primary tumor or the initial cell line. All bands revealed by the RBL5 probe can be

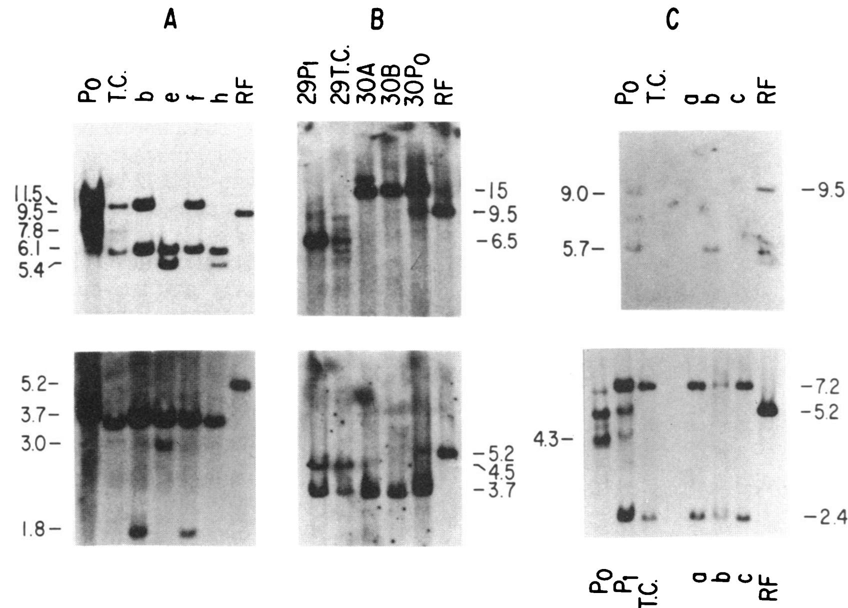

FIG. 2. Blots of *Hin*dIII fragments of DNA from the MeNU-induced RF/J T lymphomas MNU31 (*A*), MNU29 and MNU30 (*B*), and MNU28 (*C*). Fragments hybridizing with the RBL5 (*Upper*) and Jβ2A (*Lower*) probes are shown. P_0, Primary tumor; P_1, first passage *in vivo*; TC, early passage of uncloned tissue culture line; lowercase letters, clones derived from TC line; RF, RF/J liver (control). Tumors growing *in vivo* usually show faint bands of germ-line size, presumably due to non-T cells present in the tumor.

interpreted as *V–D1–J1* or *D1–J1* rearrangements. Among the Jβ2A bands, the 3.7-kb band probably represents *D2–J2* joining, and the 3.0-kb band probably represents *V–D2–J2* joining, but the 1.8-kb band is difficult to explain by the looping-out model.

MNU29. Among *Hin*dIII fragments derived from primary lymphoma MNU29 (Fig. 2*B*), only a 6.5-kb fragment hybridized with RBL5 probe, indicating a *D1–J1* or *V–D1–J1* rearrangement. The Jβ2A probe identified two bands at 4.5- and 3.7-kb, both of which may be interpreted as *D2–J2* joinings. In the cultured line derived from this tumor, the RBL5 probe identified several submolar bands in addition to the 6.5-kb band of the primary tumor, but the Jβ2A bands remained unchanged. This finding might be interpreted as a burst of recombinational activity in the *V–D1–J1* region in a tumor that was monoclonal before adaptation to growth in culture.

MNU30. Primary tumor MNU30 was divided into two parts, each of which was passaged separately *in vivo* and then used to establish independent cell lines, MNU30A and MNU30B. In this case, analysis of the primary tumor and both cell lines (Fig. 2*B*, lanes 30A, 30B, and $30P_0$) revealed identical *Tcrb* fragments: a 15-kb RBL5 fragment representing a *V–D1–J1* rearrangement and a 3.7-kb Jβ2A fragment representing a *D2–J2* rearrangement. However, cultured line 30A showed an additional RBL5 band larger than 15 kb that was not detected in other MNU30 derivatives.

MNU28. Primary tumor MNU28 already showed some signs of possible heterogeneity. The RBL5 probe identified major bands at 9.0- and 5.7-kb plus several minor bands (Fig. 2*C*, lane P_0). None of these bands appeared at more than trace levels in the initial cell line, and among three cloned derivatives of this line, only the 5.7-kb band was detectable in a single clone, indicating that the *C1* region had been completely deleted from most tumor derivatives. The Jβ2A probe revealed major bands in the primary tumor at 5.2- and 4.3-kb plus at least four minor bands at 7.2, 4.8, 3.1, and 2.4 kb. Only the minor bands at 7.2 and 2.4 kb persisted in the cell line and its clonal derivatives.

The most likely interpretations of these data are either that this tumor was polyclonal in origin or that multiple *Tcrb* gene rearrangements occurred in the descendants of the progenitor tumor cell, some of which survived only transiently. The major RBL5 components of the primary tumor showed a *D1–J1* pattern on one chromosome 6 and a *V–D1–J1* on the other. The Jβ2A bands suggest that one chromosome 6 was unrearranged in this region, whereas a *D2–J2* rearrangement had occurred on the other. During adaptation to growth in culture, the large majority of cells showed deletion of the *C1* region and *V–D2–J2* rearrangements on both chromosomes 6.

Igh Gene Studies. Because some T lymphomas show rearrangements in the region of the *Igh-1* locus for the immunoglobulin heavy chain (13, 14), we sought further evidence for mono- or polyclonality of the tumors by examining the patterns of DNA restriction fragments in these tumors and derivatives that hybridize with the J11 probe (Fig. 3 and data not shown). All samples from MNU29 showed a new band at 3.3 kb in addition to two germ-line bands at 2.3 and 1.0 kb. All samples from MNU31 showed only germ-line bands. Primary tumor MNU30 showed only germ-line bands, but all later passages (30A and 30B) from both sublines showed a new 25-kb band and loss of the 1.0-kb germ-line band. All samples from MNU28 showed a new band at 2.1 kb in addition to germ-line bands. Thus, these data suggest that the four tumors presented so far may, indeed, have been monoclonal in origin.

MNU19 and MNU27. Data from two of the tumors studied here do not provide convincing evidence for a monoclonal origin of the tumors. DNA from the second and third passages *in vivo* of MNU19 (Fig. 4*A*, lanes P_2 and P_3) showed only a faint germ-line band with the RBL5 probe (*Upper*), and the Jβ2A probe (*Lower*) revealed two non-germ-line bands at 9.0 and 5.6 kb, suggesting that most cells had deleted the *C1* region and possessed *D2–J2* rearrangements on both chromosomes. However, the MNU19 cell line and all clonal derivatives showed a strong 15-kb RBL5 band and two Jβ2A bands at 3.8 and 3.4 kb, suggesting a *V–D1–J1* and a *D2–J2* arrangement on one chromosome and a different *D2–J2* rearrangement on the other. These two sets of samples also showed two different patterns of *Igh-1* gene rearrangement with the J11 probe (data not shown). Because it is not clear how the later set of banding patterns might have arisen from the earlier set, more than one clonal population may have been present in the early passages of

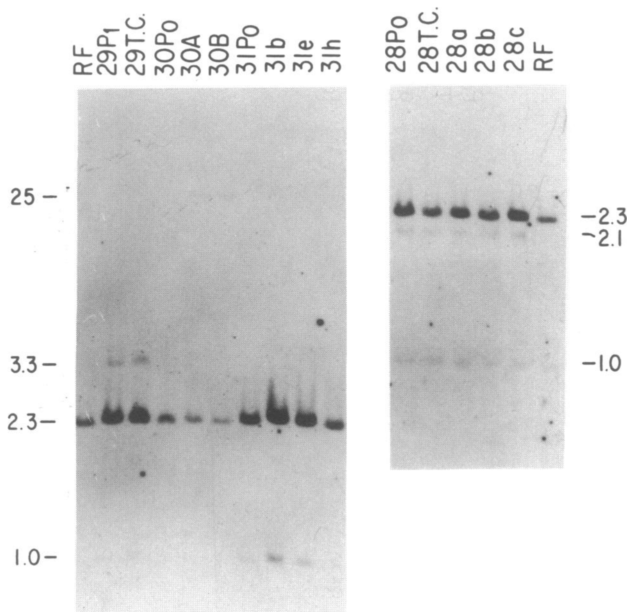

FIG. 3. Blots of *Hin*dIII fragments from samples of MeNU-induced T lymphomas 28 (*Right*), 29, 30, and 31 (*Left*) probed with J11 (*Igh-1*-specific). P_0, primary tumor; P_1, first passage *in vivo*; TC, early passage of uncloned tissue culture line; lowercase letters, clones derived from TC line; RF, RF/J liver (control). 30A and 30B were independent cell lines established from primary tumor MNU30.

the tumor, and the one that eventually predominated in cultured tumor lines was probably a minority population in the early tumor.

MNU27 is another tumor for which the *Tcrb* DNA patterns may be incompatible with monoclonal origin of the tumor. DNA from the primary tumor and from early passages *in vivo* showed only faint RBL5 bands at germ-line or greater molecular sizes (Fig. 4*B Upper*, 5 P-labeled lanes) and only a weak 4.2-kb Jβ2A band suggesting a *D2–J2* joining (*Lower*, lanes P_0 and P_3). DNA from the seventh passage *in vivo*, immediately preceding adaptation to growth in culture, revealed several new bands with both probes. After adaptation to culture, the cells displayed a strong

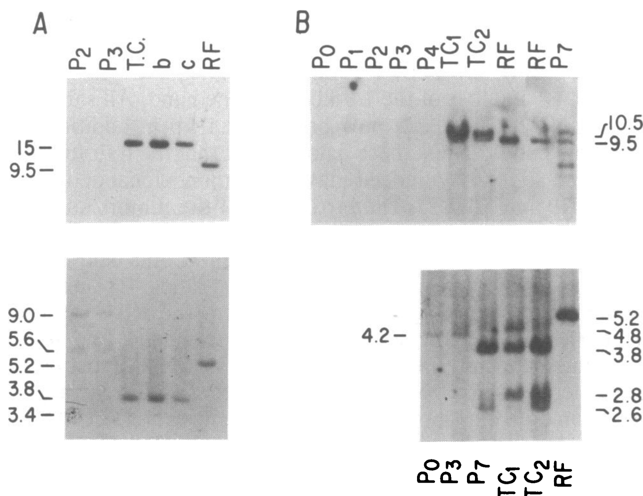

FIG. 4. Blots of *Hin*dIII fragments from samples of MeNU-induced T lymphomas MNU19 (*A*) and MNU27 (*B*). Probes were RBL5 (*Upper*) and Jβ2A (*Lower*). P_0, Primary tumor; P_{1-7}, tumor passage *in vivo*; TC_1 and TC_2, early and late passages of uncloned tissue culture lines; b and c, clones from tissue culture line; RF, RF/J liver (control).

banding pattern consisting of a subset of the fragments that were emerging in the last passage *in vivo*. DNA extracted immediately after establishment of the cultured line (TC_1) showed an RBL5 band at 10.5 kb, representing a *V–D1–J1* rearrangement, plus a fainter band of germ-line size, and this pattern remained constant after 4 mo in culture (TC_2). Experiments with the Jβ2A probe clearly suggested polyclonality in the later derivatives of the tumor because the same four bands at 4.8-, 3.8-, 2.8-, and 2.6-kb were present at different relative intensities in DNA from the last passage *in vivo* and the early- and late-cultured lines. As for MNU19, two different patterns of rearrangement in the *Igh-1* gene were evident in various derivatives of tumor MNU27 (data not shown).

DISCUSSION

Several conclusions emerge from our studies of DNA rearrangements within the *Tcrb* gene region in chemically induced lymphomas of RF/J mice. (*i*) DNA rearrangements were evident in all primary tumors examined, but there was no apparent uniformity in the patterns of rearrangement seen from one tumor to another. (*ii*) In most cases patterns observed in DNA from a primary tumor did not remain constant in cells of the same tumor during passages *in vivo* and after adaptation to growth in culture. (*iii*) The successive patterns seen during serial tumor passage could sometimes be plausibly interpreted as further rearrangements within a monoclonal tumor line, but other tumors showed successive patterns that were not easily compatible with an origin from an initially monoclonal cell population.

It is not surprising that each lymphoma examined showed an entirely different pattern of *Tcrb* gene rearrangement. It has been proposed (15) that murine T lymphomas might arise from T cells with immunologic specificity for endogenous murine leukemia viruses that might be the proximal causal agent of the chemically induced tumors. However, such viruses may well be antigenically diverse, and only if the susceptibility of T cells to malignant transformation depended on their being immunologically specific for one or a very few antigenic epitopes would receptor gene rearrangements be expected to show any degree of uniformity. No current theory of chemical lymphomagenesis includes such a stringent component.

The observation that patterns of *Tcrb* gene rearrangements in cells of individual tumors varied during serial propagation *in vivo* and in culture parallels analogous findings of new arrangements within immunoglobulin gene regions in hybridomas and tumor cells of the B-lymphocyte lineage that can occur by any of several different mechanisms (16). The data generated in our experiments with T-lymphoma lines suggest that many of the *Tcrb* gene rearrangements represented *V–D1–J1*, *D1–J1*, or *D2–J2* joinings that had not occurred earlier; in other cases it appeared that a new *V–D2–J2* rearrangement had occurred, resulting in the deletion of *C1* sequences. None of our findings suggested a mechanism analogous to the class switch that can occur in B cells; such a rearrangement would presumably involve joining of a *V–D1–J1* rearrangement with a *C2* segment plus deletion of the intervening *C1–D2–J2* regions, and we did not detect the complete deletion of the *J2* region from cells in which it had been present in earlier passages.

A definitive answer to the question of whether MeNU-induced lymphomas are generally monoclonal in origin does not emerge from our studies. Results from tumor MNU30, which showed mainly a single pattern of *Tcrb* rearrangement throughout its passage history, strongly indicate a monoclonal origin. Results from tumors MNU31, MNU29, and MNU28, which showed considerable variation in *Tcrb* rearrangement patterns during serial passage, can be tenta-

tively reconciled with the hypothesis of a monoclonal origin. However, tumors MNU19 and MNU27 may well be polyclonal in origin, although in these cases malignant transformation could have occurred in an immature T cell in which the *Tcrb* gene had undergone little or no rearrangement, thereby leaving opportunities for diverse patterns to emerge within an initially monoclonal population.

Identification of our MeNU-induced tumors as T lymphomas was confirmed by immunofluorescence studies indicating high-level expression of Thy-1, Lyt-2 and MEL-14 antigens on cells of all lines (unpublished data). It has been noted in other studies that rearrangements within immunoglobulin gene regions can occur in cells of the T-lymphocyte lineage (13, 17). DNA rearrangements in the *Igh-1* gene region were noted in some of our MeNU-induced tumors. Thus, some activity of a mechanism for immunoglobulin gene rearrangement was expressed either in these T-lymphoma cells or in their nonmalignant precursors. It is not yet known whether these presumably inappropriate rearrangements are due to (*i*) low-level expression of the as yet undefined cellular machinery for B cell-specific immunoglobulin gene rearrangement, (*ii*) an inefficient activity on immunoglobulin genes of the cellular machinery for TCR gene rearrangement, or (*iii*) leaky specificity of cellular mechanisms that direct a common rearrangement machinery to operate on the appropriate immunoglobulin or TCR genes in B or T cells, respectively. The capacity of cultured cells of the B-lymphocyte lineage to undergo further immunoglobulin gene rearrangement has permitted a number of elegant studies of mechanisms operating in this system (18). Our observations suggesting a similar capacity for further rearrangement within the *Tcrb* gene in cultured T lymphomas may lead to useful systems for analogous studies of mechanisms of TCR gene rearrangement.

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