T-Cell Lymphoma Lines Derived from Rat Thymomas Induced by Moloney Murine Leukemia Virus: Phenotypic Diversity and Its Implications

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The phenotype of 27 Moloney murine leukemia virus-induced rat thymic lymphomas and 36 cell lines derived from these tumors was determined by using 18 monoclonal antibodies directed against hematopoietic cell surface determinants. The cell lines and the primary tumors from which they were derived were clonally related as determined by the pattern of provirus integration and the pattern of rearrangement of the T-cell receptor β and δ and *Igh* loci. The differentiation phenotype of the primary tumors and the cell lines derived from them were related. The differences observed between the primary tumors and the cell lines could be explained either by the selection of subpopulations of tumor cells during establishment in culture or by the phenotypic instability of the tumor cells. One cell line (LE3Sp) underwent the transition from a CD4⁺ CD8⁺ to a CD4⁺ CD8⁻ phenotype following exposure to interleukin-2 in culture. Both the primary tumors and the cell lines derived from them express a wide range of phenotypes which correspond to multiple stages in T-cell development. This observation suggests that the pleiomorphism of retrovirus-induced lymphomas, which had been suggested previously from the analysis of mouse tumors, is an intrinsic property of the process of oncogenesis and is not due to the transformation of different types of cells by spontaneously arising leukemogenic variants of the inoculated virus. The wide spectrum of phenotypes expressed by these tumors suggests that Moloney murine leukemia virus may infect and transform T cells at various stages of development. Alternatively, the target cells may be immature T-cell precursors which, following transformation, continue to differentiate. A host of early findings, suggesting that the repertoire of target cells is restricted to poorly differentiated hematopoietic progenitors, and the ability of the LE3Sp cell line to differentiate in culture indicate that the latter possibility may be more likely. The data in this report address the extent and mechanism of the phenotypic variability of retrovirus-induced rodent T-cell lymphomas. In addition, they demonstrate the potential usefulness of the T-cell lymphoma lines we have established in studies of oncogenesis and T-cell differentiation.

The establishment of tumor cell lines in culture has been instrumental in the study of oncogenesis. The phenotypic characterization of tumor cell lines usually provides critical information about the phenotype of the primary tumors of their origin and about the nature of the target cells (38). In addition, tumor cell lines provide live specimens which can be used to determine the biological properties of the tumor cells and the genetic changes which may be responsible for these properties (2, 4a, 25a, 26, 31, 59). This report describes the establishment and phenotypic characterization of 36 cell lines derived from 27 primary T-cell lymphomas induced in rats by Moloney murine leukemia virus (MoMuLV) and explores the implications of the results for the biology of oncogenesis.

Tumors induced by a variety of RNA tumor viruses express phenotypes that are characteristic of the causative virus. Thus, tumors induced by human T-cell lymphotropic virus type I are phenotypically CD4⁺ IL-2R⁺ T cells (44). Tumors induced by bovine leukemia virus are phenotypically CD5⁺ pre-B lymphocytes (8, 17), and tumors induced by Abelson murine leukemia virus are pre-B lymphocyte progenitors (41). In contrast to these tumors, the phenotype of thymic lymphomas induced by nontransforming retroviruses in rodents appears to be variable. These tumors form a

broad group which includes spontaneous and mink cell focus-forming virus-induced thymic lymphomas in high leukemia mouse strains as well as radiation leukemia virus- and MoMuLV-induced thymic lymphomas in mice and rats (55). Different reports have suggested that spontaneous and mink cell focus-forming virus-induced tumors in AKR mice are phenotypically related to the cortisone-sensitive immature cortical thymocytes (32) or that they represent relatively mature thymocytes expressing low levels of Thy1, high levels of class I major histocompatibility (MHC) antigens, and low levels of peanut agglutinin receptors (10, 23, 67, 69). Other studies have suggested that the thymic lymphomas developing spontaneously in AKR mice represent a subpopulation of thymocytes which carry rearrangements in both the α - and the β -chain genes of the T-cell receptor (TCR) but they lack TCR expression (33). Although these studies report significantly different predominant tumor cell phenotypes, they generally agree that tumors induced by a single agent could exhibit some degree of phenotypic variability (10, 21, 28, 30, 32, 33, 40, 67, 69). However, these studies are difficult to interpret because many of them were conducted more than a decade ago, when only a limited number of T-cell differentiation markers had been recognized.

The results of these studies raise two major questions: (i) if the tumors were phenotypically pleiomorphic, what is the extent of their phenotypic variability, i.e., what is the range of the possible tumor cell phenotypes? and (ii) what is the

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TABLE 1. T-cell surface determinants of rat hematopoietic cells

Antibody	Antigenic determinant	Cellular localization	Reference or source
W3/25	CD4 (L3T4)	T-helper cells, macrophages	4, 64
OX-8	CD8 (Lyt2)	Suppressor and cytotoxic T cells	4, 7
OX-34	CD2 (T11)	T-lymphoid lineage, spleen macrophages	63
OX-19	CD5 (Ly1)	T lymphocytes	14
OX-39	IL-2R	Subset of T cells	35
OX-7	Thy1	Thymocyte glycoprotein	3
OX-6	IaA (class II)	B cells, macrophages, dendritic cells	19, 29
OX-17	IaE (class II)	Cortical epithelial and medullary reticular cells, macrophages	19
OX-18	RT-1A (class I)	Subfraction of thymocytes, peripheral T cells	19
OX-22	Leukocyte common antigen (high molecular weight)	B cells, peripheral cytotoxic T cells, and some T-helper cells; subpopulation of thymocytes	49, 65
OX-1	Leukocyte common antigen	Thymocytes	52
W3/13		All T-cell lineage in thymus and myeloid cells in bone marrow	64
OX-41	Macrophage antigen	Macrophages	43
OX-42	Complement receptor type 3	Macrophages	43
OX-43		Macrophages, endothelial cells	42
OX-44		Intermediate subpopulation of thymocytes	34, 36
MAR18.5	Surface immunoglobulin	B cells	H. Kimura

mechanism underlying the development of tumors with variable phenotypes? A hypothesis aiming to address the mechanism of the phenotypic variability was based on the finding that during the preleukemic period, high leukemia mouse strains express a theoretically unlimited number of leukemogenic virus variants (51). If each variant targets a different population of cells, the developing tumors could be pleiomorphic. To address this hypothesis, newborn mice were inoculated with retrovirus variants arising spontaneously in preleukemic animals and the phenotype of the developing tumors was examined to determine whether it was virus specific (66, 68). However, the results of two independent studies conducted along these lines were contradictory (66, 68). We hypothesize that the conflict may be due at least in part to the fact that inoculated mice continue to generate new virus variants.

This study revealed that the phenotype of both the primary thymomas induced by MoMuLV in rats and the cell lines derived from them is variable and corresponds to multiple stages in T-cell development. One cell line is phenotypically unstable if cultured in the presence of interleukin 2 (IL-2). These data address both questions posed above. Indeed, on the basis of these results, we conclude that retrovirus-induced T-cell lymphomas in rodents are unique among virus-induced tumors in that they express highly variable phenotypes. Furthermore, since MoMuLV is genetically stable during oncogenesis in rats (59), the multiplicity of tumor cell phenotypes is an intrinsic property of the process of oncogenesis and is not due to the transformation of different target cells by virus variants generated during the preleukemic period. The logical extension of these conclusions is either that the virus transforms T cells at various stages of maturation or that the target cells are immature hematopoietic cells that continue to differentiate following transformation. Given the phenotypic instability exhibited by at least some of these cells in culture, we suggest that the second possibility is more likely.

The results presented in this report provide insight into the biology of MoMuLV-induced T-cell lymphomas in rodents and suggest that the T-cell lymphoma lines we established will be very important in studies of viral oncogenesis and T-cell differentiation.

MATERIALS AND METHODS

Tumor induction and establishment of cell lines. Tumors were induced by intraperitoneal inoculation of newborn Osborn-Mendel or Long-Evans rats with 50,000 XC PFU of MoMuLV as previously described (59–61). Thymic lymphomas developed within 2 to 6 months after inoculation. Tumor cells from the thymic lymphomas were either passaged in nude mice and then put into culture or they were cultured directly. Tumor cells from rats or nude mice were cultured in RPMI 1640 supplemented with 10% horse serum, penicillin, streptomycin, and kanamycin (50 U/ml, 50 µg/ml, and 100 µg/ml, respectively), 5 mM β-mercaptoethanol, and, in some cases, 100 U of recombinant human IL-2 per ml. The concentration of tumor cells in the primary cultures was approximately 10⁷/ml.

The cell lines were given the number of the primary tumor of origin. When multiple sublines were derived from a single primary tumor, they were given the name of the tumor followed by a numbering letter (A, B, C, etc.).

Cell surface characterization. Both primary tumors and cell lines were examined for the expression of surface markers detected by 18 monoclonal antibodies (MAbs) directed against rat hematopoietic cell surface determinants (Table 1). A total of 10⁶ cells were washed in phosphate-buffered saline with 3% fetal calf serum and incubated with the specific MAb for 30 min at 4°C. After being washed, the cells were stained with a fluorescein-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulin G antibody in a similar way. The second antibody alone was used as a negative control to determine nonspecific binding. The cells were suspended in phosphate-buffered saline with 3% fetal calf serum and propidium iodide to stain nonviable cells. The cells were analyzed by flow cytometry using a FACStar Plus instrument (Becton-Dickinson and Co., Paramus, N.J.). The purpose of using a combination of different cell surface markers was to define both the lineage and the developmental stage of the cells.

DNA preparation and Southern blot analysis. Genomic DNA from primary thymic lymphomas and cell lines was prepared as previously described (26, 42, 59). For Southern blots, 10 µg of DNA was digested with restriction endonu-

cleases under the conditions suggested by the manufacturer (Bethesda Research Laboratories, Inc., or New England BioLabs, Inc.). DNA was transferred and cross-linked by UV irradiation to nylon membranes (Hybond-N from Amersham International). Hybridization to a MoMuLV long terminal repeat probe and to pRI15 (19), a probe derived from the J region of the rat immunoglobulin heavy chain (*Igh*) locus, was performed under high-stringency conditions. Hybridization to probes from the J β 2 region of the murine T-cell receptor β was performed as previously described (5). Finally, hybridization to a probe from the J region of the δ locus (11) was performed in the presence of 40% formamide-6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C for 18 h. The blot was washed in 2 $\times$ SSC-0.1% sodium dodecyl sulfate at 65°C for 30 min and in 0.2 $\times$ SSC-0.1% sodium dodecyl sulfate at 65°C for an additional 30-min period.

Immunohistochemistry. Indirect immunofluorescence (13) was performed by using 5- μ m-thick frozen sections from 27 primary rat T-cell lymphomas. After the sections were mounted on glass slides, they were fixed for 10 min in cold acetone, washed with phosphate-buffered saline, and incubated at room temperature for 1 h with a 1/20 dilution of the MAbs W3/13, W3/25, OX-8, OX-34, OX-19, OX-39, OX-18, OX-1, OX-44, OX-7, and OX-17 (Table 1). Affinity-purified fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used in a 1/20 dilution as the secondary antibody. After incubation with the secondary antibody for 1 h, the sections were washed in phosphate-buffered saline, mounted in 10% glycerin, and examined in a fluorescence microscope. Normal spleen and lymph node sections were used as positive controls. All sections were also processed without the primary antibody to assess background nonspecific immunofluorescence.

RESULTS

The phenotypes of the MoMuLV-induced rat T-cell lymphoma lines correspond to multiple stages in T-cell development. All the cell lines established in tissue culture were stained by using the panel of 18 MAbs described in Materials and Methods, and they were analyzed by flow cytometry to determine their differentiation phenotypes. A representative sample of the results showing the patterns of reactivity of six MAbs (recognizing W3/13, CD2, CD5, CD4, CD8, and IL-2 receptor [IL-2R]) against three tumor cell lines (D₁, 6922B, and 2788) is shown in Fig. 1. These cell lines appear to define three successive stages in T-cell development. The pattern of reactivity of three additional MAbs (OX-1, OX-22, and OX-44) with the same cell lines, shown in Fig. 2, may define cell subpopulations within these developmental stages.

Table 2 shows a summary of the data. The cell lines were ordered along the pathway of T-cell development on the basis of the expression of five markers (CD2, CD5, Thy1, CD4, and CD8) which are known to be expressed in a stepwise fashion during T-cell development (45). Two of the MAbs, W3/13, which defines an antigen not yet characterized, and OX-1, which defines an antigen expressed on the molecule of the leukocyte common antigen (LCA), reacted with all the cell lines. The only exception was the cell line 6922A which was negative for OX-1. This is consistent with the finding that the antigens detected with these antibodies represent general markers of the T-cell lineage and are expressed in all stages of T-cell development (52, 64). All cell lines were negative for antibodies MAR18.5, OX-41, OX-42, and OX-43 (data not shown).

FIG. 1. Fluorescence-activated cell sorter analysis of cell lines D₁, 6922B, and 2788 with MAbs for the major T-cell differentiation markers.

The antigenic determinants CD2, CD5, and Thy1 are expressed early in T-cell development, and they may be involved in this process (45). CD2 induces cell proliferation following interaction with the appropriate MAbs or with the leukocyte function-associated antigen 3 which may be its normal ligand (46). Similarly, CD5 induces cell proliferation following interaction with the appropriate MAbs (14), although its normal ligand remains unknown. Thy1 is a member of the immunoglobulin gene superfamily (62). Polyvalent antibodies against this molecule induce IL-2 expression in some T-cell tumor lines (47). Furthermore, although the normal function of Thy1 remains undetermined, its aberrant expression in transgenic mice leads to the development of kidney adenocarcinomas (24). On the basis of the expression of these antigens, the cell lines can be classified in groups corresponding to successive stages of thymocyte differentiation. Detection of these antigens in cell lines, which do not express other differentiation-dependent T-cell markers, defines a set of cell lines that represent an early stage in T-cell development.

Two of the MAbs, W3/25 and OX-8, recognize the CD4 and CD8 molecules which are used to identify the major functional populations of mature T cells, i.e., the helper and the cytotoxic cells. Both molecules are involved in antigen recognition. This is mediated by interactions with the class II

FIG. 2. Fluorescence-activated cell sorter analysis of cell lines D₁, 6922B, and 2788 with MAbs OX-1 and OX-22 (defining different antigenic determinants of the LCA) and OX-44. This analysis reveals heterogeneity between tumors with similar differentiation phenotypes.

or class I MHC molecules on the surface of antigen-presenting cells (CD4 and CD8, respectively) (53). The cell lines derived from the MoMuLV-induced rat T-cell lymphomas express CD4 and CD8 in all possible combinations, as expected for cells corresponding to different stages of T-cell differentiation. However, the frequency of cell lines representing these combinations is interesting. Thus, the CD4⁺ CD8⁺ phenotype, which is expressed in 85% of the thymocytes (20, 45), is represented by only two cell lines (Table 2). Furthermore, the CD4⁺ CD8⁻ and CD4⁻ CD8⁺ phenotypes are represented by 1 and 11 cell lines, respectively. This is surprising, because the last two phenotypes are present in only 12% of the normal thymocytes and are represented almost evenly in the normal rat thymus (20).

The IL-2R was expressed in 10 cell lines, of which 8 were derived from IL-2R primary tumors (Tables 2 and 3). Expression of IL-2R was observed in six cell lines established in culture in the absence of IL-2 (2770, LE3Sp, 2788, 2775, H41, and 2789) and four cell lines which were established in the presence of IL-2 (they are IL-2 dependent) (5677, 5677.6, 2780, and 4437). It is interesting that one cell line (5675.1) depends on IL-2 for growth, although it does not express detectable levels of IL-2R, a phenomenon consistent with previous findings from other laboratories (48, 56). This may be due to the interaction of IL-2 with the IL-2R β molecules which are constitutively expressed in T cells (27, 39). The overall pattern of expression of IL-2R in the cell lines is consistent with the finding that this molecule may be expressed only in the late stages of thymic differentiation in the rat (54), contrary to the mouse, in which a significant number of thymocytes appear to be IL-2R⁺ (9, 27, 37, 39).

The marker defined by the MAb OX-44 was detected in a random fashion in the cell lines. This is consistent with the finding that this antigen is expressed in subpopulations of thymocytes at all stages of T-cell development. One particular phenotype (CD4⁺ CD8⁺ OX-44⁺) represented by the

cell line LE3Sp defines a thymocyte subpopulation in the rat (34, 36).

The expression of class I MHC was determined by using the MAb OX-18. The expression of class II MHC was determined by using two antibodies, OX-6 directed against IaA determinants and OX-17 directed against IaE determinants. None of the cell lines scored positive for class II MHC molecules. On the contrary, all the cell lines expressed class I MHC. Two-thirds of the cell lines have the phenotype CD4⁻ CD8⁻ OX-18⁺ which has been demonstrated in 2% of normal thymocytes (19). The phenotype CD4⁺ CD8⁺ OX-18⁻, which is observed in about 70% of normal thymocytes (19), was not detected in these cell lines.

OX-22 is a MAb which recognizes an antigenic determinant of the LCA, a family of surface glycoproteins, which is expressed in B cells, in some thymocytes, in CD4⁻ CD8⁺ cells, and in a subpopulation of CD4⁺ CD8⁻ T cells (49, 57). The expression of different LCA determinants at different stages of T-cell maturation depends on the expression of different forms of the LCA protein which in turn is controlled by differential splicing or posttranslational modifications such as glycosylation (57). A total of 13 cell lines scored positive for OX-22 expression. However, expression of OX-22 did not follow a clear developmental pattern. This may be due to the fact that similar to OX-44 (34), the OX-22 LCA marker may be expressed in subpopulations of thymocytes at multiple stages of T-cell development (25). Alternatively, this may be due to tumor-specific effects on the differential splicing of the LCA message or on the pattern of posttranslational modifications in the LCA protein.

The T-cell markers described above are not expressed randomly in the T-cell lymphoma lines. The hierarchical order of expression of these markers indicates that each cell line corresponds to a specific stage of T-cell development. This is shown in Table 1 and diagrammatically in Fig. 3. Some of the cell lines were not included in this figure because of the aberrant expression of one or more differentiation markers. For example, the cell line 2765C was not included because, although it is composed of differentiated CD4⁺ CD8⁻ cells, it does not express the CD2 marker. The aberrant expression of differentiation markers may be due to the process of transformation. Alternatively, it may define subpopulations of thymocytes not yet characterized.

In some cases, multiple sublines were established from a single primary tumor. Figure 3 shows that these sublines differ only slightly, suggesting that they may represent consecutive steps of T-cell development. These observations also suggest that the cell lines are accurate representatives of the primary tumors and that the tumor cells undergo limited differentiation during establishment in culture. To determine the relationship of the cell lines with the primary tumors from which they were derived, we examined whether they were clonally related and we established the immunophenotype of the primary tumors.

Clonal relationship between cell lines and the primary tumors. The 36 cell lines described here were derived from 27 independent primary thymic lymphomas. To determine the clonal relationship of these cell lines and the primary tumors from which they were derived, genomic DNA from the thymic lymphomas and the derivative cell lines was analyzed by Southern blotting and hybridization to the MoMuLV long terminal repeat, TCR β and δ , and *Igh* probes.

A representative sample of the results showing the clonal relationship between three tumors (D₁, 6922, and 2788) and their derivative cell lines is presented in Fig. 4. The patterns

TABLE 2. Differentiation phenotype of MoMuLV-induced rat T-cell lymphoma cell lines^a

Cell line	Antigenic determinant												
	CD4	CD8	W3/13	Thy1	CD2	CD5	OX-22	IL-2R	RT-1A	IaA	IaE	OX-44	OX-1
5679	—	—	++	—/+	—	—	—	—	++	—	—	+	++
5681	—	—	+	—	—	—	+	—	++	—	—	+	++
D ₁	—	—	++	—	+	—	—	—	++	—	—	+/-	++
B2b	—	—	++	—	+	—	+	—	++	—	—	—	++
6889	—	—	++	—/+	++	—	+	—	++	—	—	—	++
2770	—	—	+	—	+	—	—	+	+	—	—	+	+
6922A	—	—	+	—	++	—	—	—	+	—	—	—	—
6924A	—	—	+	—/+	+/-	—	—/+	—	+/-	—	—	+	+/-
2782	—	—	++	++	—	—	+	—	++	—	—	++	++
6922B	—	—	++	++	+	—	++	—	+	—	—	—	++
2772A	—	—	+	—	+	+	—/+	—	+	—	—	+	—/+
2772B	—	—	++	—	+	+	—	—	+	—	—	++	+
2772C	—	—	+	—/+	+	+	—	—	+	—	—	—/+	+
5668	—	—	++	—	++	+	—	+	+	—	—	—	++
D ₀	—	—	++	—/+	+	+	—	—	+/-	—	—	+/-	+
6922C	—	—	+/-	—/+	+	+	—/+	—	+/-	—	—	+/-	+
2771	—	—	++	++	—	+	—	—	+	—	—	++	++
2765A	—/+	—	++	++	—	++	+	—	++	—	—	++	+
2765B	—/+	—	++	+	—	+	—	—	+	—	—	++	+
2772D	—	—	++	++	+	++	—	—	++	—	—	++	+
6922D	—	—	+	+	+	+	+	—	+	—	—	+/-	+
6924B	—	—	++	—	++	+	+	—	++	—	—	—/+	++
2774	—	—	++	—/+	+	++	—	—	++	—	—	+	++
LE3Sp	++	++	++	++	+	++	++	+	++	—	—	++	++
2788	+	++	++	++	++	++	—	+	+	—	—	—	++
2765C	+	—	+	++	—	+	—	—	++	—	—	++	+
2779	—	++	+	—/+	++	+	+	—	+	—	—	+	++
2769	—	+	+	++	+	+	+	—	++	—	—	—	++
5675.1	—	+	+	+	—	+	+	—	++	—	—	++	++
6890	—	+	++	++	+	+	—	—	+	—	—	—	+
5677	—	++	++	++	++	+	+	+	++	—	—	++	++
5677.6	—	+	+	+	+	+	+	+	+	—	—	++	++
2780	—	++	++	++	+	+	—	+	++	—	—	++	++
2775	—	+	+	++	+	+	—	+	++	—	—	+	++
H41	—	+	++	++	+	+	—	+	++	—	—	—	++
4437	—	+	++	+	+	—	—	+	+	—	—	—/+	+
2789	—	+/-	+	+	+	+	—	+/-	++	—	—	+	++

^a The symbols —/+ and +/- indicate mixed populations of positive and negative cells. The symbols + and ++ indicate positive staining. The intensity of immunofluorescence is at least ten times higher in ++ than in + cells.

of both the provirus insertion (Fig. 4A) and the TCR β rearrangement (Fig. 4B) show that the primary tumor and the derivative cell lines are clonally related. The TCR δ locus was detected in the germ line configuration in the primary tumor D₁, while in the D₁ tumor cell line, both alleles were deleted. If the TCR δ locus in the rat is localized in the region which is deleted during rearrangement of the TCR α locus, as it is seen in mice and humans (58), these data suggest that establishment in culture selected for cells carrying rearrangements of both alleles of the TCR α locus. In tumor 6922, this locus was detected in both the germ line and a rearranged form, while in the derivative cell lines only the rearranged allele was detected, implying that the nonrearranged allele was deleted. The tumor 2788 and its derivative cell line have deleted both alleles of the TCR δ locus (Fig. 4C). The deletion of both alleles of the TCR δ locus was observed in 80% of both tumors and cell lines. The detection of TCR β - and TCR α -locus rearrangements in the same tumors suggests that these tumors may express functional TCR molecules on the cell surface. Preliminary characterization of some cell lines with the MAb R73 against rat TCR α : β (22) detected the presence of TCR α : β antigens on the surfaces of cell lines with a CD4⁺ CD8⁺ phenotype and in some CD4⁺ CD8⁺ cell lines, all of them with a deletion of the δ chain (unpublished observations).

Another clonal marker used to determine the relationship between the primary tumors and the derivative cell lines was the rearrangement of the immunoglobulin heavy-chain locus. This was determined by Southern blot analysis of *Eco*RI-digested tumor and cell line DNA and hybridization to a probe derived from the J_H region (18). The example in Fig. 4D shows a cell line (2788) which carries a clonal rearrangement of the *Igh* locus. The low intensity of the rearranged *Igh* band in the primary tumor indicates that establishment of this cell line in culture was associated with the selection of a subpopulation of tumor cells carrying this rearrangement.

Differentiation phenotype of primary thymic lymphomas. Frozen tissue sections from 27 primary thymic lymphomas induced by MoMuLV were stained with 11 MAbs directed against the following cell surface markers: CD4, CD8, W3/13, Thy1, CD2, CD5, IL-2 receptor, RT-1A (class I MHC), IaE (class II MHC), and the determinants recognized by the MAbs OX-22, OX-1, and OX-44 (Table 2). A representative sample of the results showing the reactivity of three MAbs (recognizing W3/13, CD4, and CD8) against three primary tumors (D₁, 6922, and 2788) is shown in Fig. 5. The pattern of antibody reactivity in all the tumors we tested is shown in Table 3. All the thymomas were positive for the MAbs W3/13 and OX-7 (Thy1) which are specific for the T-cell lineage. When an antibody gave a mixed pattern of staining,

TABLE 3. Immunohistochemical evaluation of the differentiation patterns of MoMuLV-induced rat thymic T-cell lymphomas^a

Cell line	Antigenic determinant											
	CD4	CD8	W3/13	Thy1	CD2	CD5	OX-22	IL-2R	RT-1A	IaE	OX-44	OX-1
5679	+/-	+	+	+	+	-	-	-	+	-	+	+
5681	-	-	+	+	+	+	+/-	-	+	-	+	+
D ₁	-	-	+	+	-	-	+/-	-	-	-	+/-	+/-
B2b	-/+	+/-	+	+	+	+/-	-	-	-	-	+/-	+
6889	-	ND	+	+	+	-	-	-	+	-	+/-	+
2770	-/+	+	+	+/-	+	-	+/-	-	+	-	+/-	+/-
6922	-/+	-	+	+	+	-	-	-	+/-	-	-	+/-
6924	-	-	+	+	+	+	-	-	+	-	+	+/-
2782	-/+	-	+	+	+/-	-	-	-	+	-	+	+/-
2772	-	-	+	-	+	-	-	-	+	-	+	-
5668	+/-	+	+	+	+	-	-	-	+	-	+	+
D ₀	-	+	+	+	+	+	-	-	-	-	+/-	+
2771	-	-	+	+	+	+/-	-	-	+/-	-	+	+
2765	-	-	+	+	-	-	-	-	+/-	-	+	-
2774	-	+/-	+	+	+	+/-	-	-	+/-	-	+/-	+/-
LE3Sp	+	+	+	+	+	+	-	+	-	-	+	+
2788	-	+	+	+	+	+/-	-	-	+/-	-	-	-
2779	-	+	+	+	+	+/-	-	-	+	-	+	+
2769	-	+	+	+	+	+	-	-	-	-	+/-	+/-
5675	+	+/-	+	+	+/-	+/-	-	-	+	-	+	+
6890	-	ND	+	+	+	-	-	-	+	-	-	+/-
5677	+/-	+	+	+	+	+/-	-	-	+	-	+	+
2780	-	-	+	+	+	+/-	-	-	+	-	-	+
2775	-	-	+	+	+	-/+	-	-	+	-	-	+/-
H41T	+/-	+/-	+	+	+	+	-	-	-	-	+/-	+/-
4437	+/-	+	+	+	+	-	-	+/-	+	-	+	+
2789	-	+/-	+	+	+/-	-	-	-	+/-	-	+	+

^a The symbols -/+ and +/- indicate mixed populations of positive and negative cells. ND, Not done.

FIG. 3. T-cell lymphoma lines can be classified in groups expressing different sets of differentiation markers and corresponding to successive stages of thymocyte differentiation. Some cell lines fit only partially in this scheme. These lines may represent branchpoints of development which are not yet characterized, and they are not included in this figure. The first three differentiation steps are represented by cells which express very low levels of Thy1. The depiction of these early steps of thymocyte differentiation as Thy1⁻ reflects the low to undetectable levels of this molecule in early thymocytes. The IL-2R in the rat thymus is expressed only in a small fraction of medullary thymocytes which appear to belong to the single positive thymocyte subset (54). This subset may be represented by the tumors classified under the late pre-T differentiation stage. Pro-T, Prothymocyte.

FIG. 4. (A) Patterns of provirus insertion in thymic lymphomas (T) and cell lines (CL) D1, 6922B, and 2788. The DNA was digested with *EcoRI* and hybridized to a MoMuLV probe. (B) Rearrangements of the TCR β -chain locus in the same tumors and cell lines. The DNA was digested with *SstI* and hybridized to a probe derived from the murine J β 2 region (5). (C) Rearrangements and deletions of the TCR δ locus. (D) Rearrangements of the *Igh* J region. N, Normal rat liver DNA.

the positive cells were usually evenly distributed throughout the tissue section.

The phenotype of the primary thymomas, similar to the phenotype of the cell lines, is also heterogeneous and corresponds to different stages in T-cell development. The heterogeneity observed within individual primary thymomas for some differentiation markers is consistent with the idea that the MoMuLV-induced thymoma cells are capable of limited differentiation. The possibility that the heterogeneity was due to the interspersions of normal cells among the tumor cells is ruled out because of the homogeneous staining with some of the MAbs.

Phenotypic correlation between cell lines and primary tumors. A summary of the phenotypic correlation between the primary tumors and their derivative cell lines is shown in Fig. 6. In most cases, the cell lines and the primary tumors of origin are clearly related. Differences between them could be due to culture selection of a subpopulation of cells detectable in the usually heterogeneous primary tumor. When the phenotypes of the cell line and the primary tumor of origin differ, the cell line usually exhibits the less differentiated phenotype, suggesting that the less differentiated cells may have a growth advantage in culture. However, in some cases, the cell lines exhibit a more mature phenotype than the one observed in the primary tumor. This was observed in the cell lines derived from tumors 2780 and 2775

and suggests again that the T-cell lymphomas may retain the ability for limited differentiation.

In some cases, the difference between primary tumors and cell lines is characterized by variation in the expression of individual antigenic determinants. The most striking example of this phenomenon involved the expression of RT-1A (MHC class I gene). While all the cell lines scored positive for this determinant, seven primary tumors scored negative and an additional eight were composed of mixtures of positive and negative cells (Tables 2 and 3). The differences between primary tumors and cell lines suggest that immune selection mechanisms operating only *in vivo* favor the growth of cells that lack or have low levels of class I MHC expression.

IL-2-dependent phenotypic changes in LE3Sp cells. Several observations reported here suggested that MoMuLV-induced T-cell lymphomas retain the ability to differentiate. To test this hypothesis, we examined the differentiation phenotype of the cell line LE3Sp maintained under different culture conditions. The cell line LE3Sp expresses an intermediate differentiation phenotype when maintained under our standard culture conditions. To determine whether different culture conditions affected the phenotype, the LE3Sp cells were cultured in the presence of IL-2 for 2 weeks. Alternatively, they were inoculated intravenously into SCID mice (10^6 cells per mouse; (6), and 10 days later they were recovered and put in culture for 4 days with or without IL-2. The results shown in Fig. 7 indicate that IL-2 is associated with a shift from a CD4⁺ CD8⁺ toward a CD4⁺ CD8⁻ phenotype (Fig. 7B). This shift was more noticeable in the cells passaged in SCID mice (Fig. 7D). After treatment with IL-2, the cultures containing mixed populations of cells were sorted by staining with MAb OX-8 which detects the CD8 antigenic determinant. Consequently, we obtained two cell lines which differed in the expression of the CD8 marker (Fig. 7E and F). Similar results were obtained by sorting the cells which were passaged through SCID mice before IL-2 treatment. After being sorted, the cells were divided into two cultures and maintained either with or without IL-2. Since there was no detectable effect of IL-2 after sorting, we suggest that IL-2 is required to produce the phenotypic change but is not required for its maintenance. These results suggest that IL-2 either induced the observed phenotypic changes or selected a subpopulation of CD4⁺ CD8⁻ cells already in existence in the primary cultures. Since IL-2 appears to have no effect on the growth of the CD4⁺ CD8⁻ cells, sorted out from mass cultures of the IL-2-treated cell line, we suggest that IL-2 may be responsible for the induction of the differentiated phenotype.

DISCUSSION

The primary T-cell lymphomas induced by MoMuLV in rats, and the cell lines established from them, are clonally related. This conclusion was based on the similarity of the patterns of provirus insertion and the similarity of T-cell receptor and *Igh* rearrangements between the primary tumors and the derivative cell lines (Fig. 4). In some cases, establishment in culture involved the selection of a subpopulation of cells present in the primary tumor. This is illustrated in Fig. 4, which shows a cell line (2788) carrying a clonal rearrangement in the *Igh* locus, which was present only in a subpopulation of cells in the primary tumor.

The differentiation phenotype of the cell lines is related to the differentiation phenotype of the primary tumor from which they were derived (Fig. 6). In some cases, the cell line

FIG. 5. Immunofluorescence analysis of the primary thymomas D₁ (a, b, and c), 6922 (d, e, and f), and 2788 (g, h, and i) stained with MAb W3/13 which is a marker of the T-cell lineage (c, f, and i) and MAbs, OX-8 and W3/25, which recognize the CD8 (b, e, and h) and CD4 markers (a, d, and g), respectively. Note that in panels a, b, e, and g, some cells exhibit positive immunofluorescence, probably representing normal host lymphoid cells or dendritic cells or both.

was homogeneous with regard to differentiation markers for which the primary tumor was heterogeneous. In other cases, a cell line either lost or gained the expression of a differentiation marker by comparison with the primary tumor from which it was derived.

At least two possible mechanisms may account for the observed differences in phenotype between the primary tumor and the cell lines. Since the selective forces operating in vivo and in vitro may be different, the culture conditions may select for subpopulations of cells present in the primary tumor. This is the most likely explanation for the establishment of cell lines which are homogeneous regarding differentiation markers for which the primary tumors were heterogeneous. This mechanism is supported by the finding presented in Fig. 4D showing that establishment of tumor 2788 in culture was associated with the selection of a subpopulation of cells already in existence in the primary

tumor (which carried a rearrangement in the *Igh* locus). Alternatively, establishment in culture may be associated with nonspecific loss of expression of specific differentiation markers or with changes in the differentiation phenotype of the tumor cells. A possible example of the loss of expression of a differentiation marker was observed in tumor D₁. This tumor had a Thy1⁺ phenotype and carried a rearranged TCR β locus. The cell line established from this tumor has a Thy1⁻ phenotype, although it continues to carry the same TCR β -locus rearrangement. The ability of the tumor cells to undergo stable phenotypic changes was suggested by the phenotypic heterogeneity of the cells of the primary tumors and by the finding that the differentiation phenotype of multiple sublines derived from a single primary tumor usually represented a gradient of differentiation steps. Direct evidence for the ability of the tumor cells to differentiate was obtained by exposing the cell line LE3Sp to IL-2. This

FIG. 6. Phenotypic correlation between primary tumors and cell lines. Open squares mean identity between cell line and primary tumor. Shaded squares indicate the presence of a mixed population of cells in the primary tumor, one of which was selected during establishment in culture. Alternatively, the cell line is heterogeneous, containing at least two cell populations, one of which could be detected in the primary tumor. Closed squares indicate a difference between the primary tumor and the derivative cell line.

treatment was associated with the stable transition of the LE3Sp cells from an intermediate CD4⁺ CD8⁺ to a more differentiated CD4⁺ CD8⁻ phenotype.

The operation of different selective pressures in animals and in culture was shown by the analysis of the primary tumors and the cell lines for the expression of RT-1A. RT-1A, a determinant of class I MHC, was detected by the MAb OX-18. Of 27 tumors tested, 12 were either OX-18⁻ or

they were composed of mixtures of OX-18⁻ and OX-18⁺ cells. In contrast to the primary tumors, all the cell lines expressed high levels of the RAT-1A class I MHC determinant. Since antigen recognition occurs in the context of class I MHC (53), tumor cells expressing this marker may be subject to immune surveillance. The difference between primary tumors and cell lines may be because the immune selection ceases to operate after the cells are put in culture.

FIG. 7. IL-2-dependent phenotypic changes in LE3Sp cells. (A) Control LE3Sp cells cultured under standard conditions. (B) Cells cultured in the presence of IL-2 for 2 weeks. (C) Cells grown in SCID mice for 10 days and then cultured without IL-2 for 4 days. (D) Cells passaged in SCID mice and then cultured in the presence of IL-2 for 4 days. The CD4⁺ CD8⁻ and CD4⁺ CD8⁺ cell populations, shown in panel B, were sorted on the basis of the expression of the CD8 marker (E and F, respectively). The phenotype of these cells is stable following culture in the presence or absence of IL-2.

The differentiation phenotype of both the primary tumors and the cell lines derived from them varies from that of poorly differentiated to that of almost terminally differentiated cells. The cell lines are phenotypically more homogeneous than the primary tumors and can be ordered along the pathway of T-cell development on the basis of the expression of the Thy1, CD2, CD5, CD4, CD8, and IL-2R antigens which are expressed sequentially during normal thymocyte differentiation (Fig. 3). The variability of the phenotype of retrovirus-induced thymic lymphomas had been suggested previously from the analysis of retrovirus-induced mouse tumors. However, the extent and the mechanism of this variability had not been determined. The results presented here address both these questions. Thus, our data showing that MoMuLV-induced T-cell lymphomas in rats express phenotypes that extend through the spectrum of T-cell differentiation provide the most comprehensive evaluation to date of the range of possible phenotypes for this type of neoplasm. Furthermore, since MoMuLV is genetically stable during oncogenesis in rats (59), these data indicate that the phenotypic variability among retrovirus-induced rodent thymic lymphomas is a property intrinsic to the process of oncogenesis and that it is not due to the transformation of

different target cells by leukemogenic virus variants generated during the preleukemic period (68). We conclude that MoMuLV and other murine nontransforming retroviruses are unique among viruses in that they induce neoplasms with highly variable phenotypes. The phenotypic variability of retrovirus-induced lymphomas is reminiscent of the variability observed in human lymphoid neoplasms (20), which is surprising, because the rodent thymic lymphomas, contrary to the human lymphoid tumors, are induced by a single etiologic agent.

The frequency of the T-cell differentiation phenotypes in the MoMuLV-induced T-cell lymphoma lines differs markedly from the frequency of these phenotypes among normal rat thymocytes (20). The most striking example of a phenotype detected at an unexpected high frequency is the CD4⁻ CD8⁺ phenotype which was present in 11 cell lines. These cells could represent either differentiated late pre-T cells or a subset of early thymocytes which are OX-44⁻ CD3⁻ and carry unrearranged TCR loci (36). However, although some of the CD4⁻ CD8⁺ cell lines are OX-44⁻, they all carry TCR rearrangements and they express a TCR β -cell surface antigen (unpublished data), suggesting that they represent late pre-T cells. The high frequency of the CD4⁻ CD8⁺ phenotype among these cell lines may be due to the viral tropism, or alternatively, it may reflect selective pressures characteristic of the tissue culture conditions. The fact that a similar preference for the CD4⁻ CD8⁺ phenotype was also observed for the primary tumors (Table 1) makes it unlikely that the high frequency of clonal selection of CD4⁻ CD8⁺ cells among MoMuLV-induced rodent lymphomas is a tissue culture phenomenon.

The phenotypic heterogeneity of MoMuLV-induced T-cell lymphomas suggests that (i) MoMuLV infects and transforms cells at all stages of T-cell development or (ii) the virus transforms immature T lymphocytes which undergo limited differentiation during oncogenesis. Our findings, which indicate that the tumor cells are phenotypically unstable, provide strong support for this last suggestion. This interpretation is in agreement with the conclusions from earlier studies addressing the nature of the target cells. These studies had shown that virus inoculation in newborn animals was followed by the accumulation of immature hematopoietic cells in the bone marrow and spleen, leading to splenic hyperplasia (15, 16, 50). Adoptive transfer experiments had shown that the bone marrow and spleen of these animals contain cells committed to neoplastic transformation (1). Since the majority of the cells in the bone marrow and spleen of the inoculated animals are phenotypically immature, it is likely that the partially transformed cells identified in these experiments belong to an early stage in T-cell development and that tumor induction is linked to the differentiation of one or more clones of these cells in the thymus. In separate experiments, it was shown that the great majority of the infected cells in young AKR mice, inoculated with lymphomagenic mink cell focus-forming viruses, were immature cortical thymocytes (12, 32). The logical extension of the conclusion that the cells targeted by the virus are immature T cells is that differentiation in the thymus may be an important component of the process of oncogenesis.

The data presented in this report provide significant insight into the range and mechanism of the phenotypic heterogeneity of retrovirus-induced rodent T-cell lymphomas and into the potential nature of the cells targeted by the virus. In addition, the cell lines presented here are expected to be valuable reagents in studies of oncogenesis and T-cell differentiation.

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