Murine T-Lymphomas Corresponding to the Immature CD4^-8^ Thymocyte Subset

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N-methyl-N-nitrosourea induces murine CD4^-8^ T-lymphomas that express high levels of J11d and low levels of CD5 antigens, a phenotype characteristic of immature CD4^-8^ thymocytes. This assignment is supported by the fact that CD4^-8^ lymphoma cell lines acquire CD4 expression after intrathymic (i.t.) transfer, a finding consistent with the established precursor potential of the normal immature CD4^-8^ subset. CD4^-8^ lymphomas recovered after i.t. transfer maintain a CD4^-8^ phenotype in long-term culture. Northern blot analyses reveal that CD4 expression is regulated at the transcriptional level in immature CD4^-8^ and CD4^-8^ cell lines. CD4^-8^ lymphomas express low levels of functional CD3/TCR complexes that mediate intracellular Ca2^+ mobilization in response to CD3 or α/β-TCR monoclonal antibody. These data suggest that the immature CD4^-8^ subset contains cells capable of undergoing TCR-mediated signaling and selection events. In contrast to normal immature CD4^-8^ cells, which comprise a heterogeneous and transient subset, the CD4^-8^ lymphoma lines provide stable, monoclonal models of the immature CD4^-8^ stage of thymocyte development.

KEYWORDS: T-cell development, T-lymphoma, thymocyte subsets.

INTRODUCTION

T-lymphocytes mature within the thymus, which provides the appropriate cellular and humoral microenvironment to induce differentiation of immature precursors into functional T-cells (Adkins et al., 1986). Normal adult thymocytes are classically grouped into four populations based on expression of CD4 and CD8 accessory molecules. The CD4^-8^ subset contains intrathymic precursors that give rise to other thymocyte subsets in irradiated hosts or in organ culture (Fowlkes et al., 1985). The immature CD4^-8^ subset constitutes the major thymocyte subset. Most CD4^-8^ cells express low levels of clonotypic α/β-TCR and are destined to die in situ presumably as a result of negative selection or failure to undergo positive selection (McPhee et al., 1979; Havran et al., 1987; Richie et al., 1988). CD4^-8^ and CD4^-8^ medullary cells express high levels of α/β-TCRs and are functionally, as well as phenotypically, similar to thymic emigrants and peripheral T cells (Ceredig et al., 1982; Scollay et al., 1984).

Although the classification scheme based on CD4 and CD8 expression has been useful in studying the relationships among thymocyte subsets, it is increasingly evident that considerable heterogeneity exists within each subset. For example, the CD4^-8^ compartment contains cells that differ in antigen expression as well as in precursor potential (Adkins et al., 1986). Recent studies have demonstrated that the CD4^-8^ subset also is heterogeneous in anatomical location, α/β-TCR expression and functional potential. In addition to mature medullary CD4^-8^ cells, a distinct subset of functionally immature CD4^-8^ cells in the adult and neonatal thymic cortex is characterized by undetectable or low levels of CD3 expression and high levels of heat-stable antigen (HSA) expression (Crispe and Bevan, 1987; Ceredig, 1988). This subset represents a transient population of cycling thymocytes that is intermediate between CD4^-8^ and CD4^-8^ maturation stages (Penit and Vasseur, 1988). Acquisition of CD4 expression occurs on immature CD4^-8^ cells after intrathymic transfer or in vitro
incubation (MacDonald et al., 1988; Guidos et al., 1989).

Due to the transient nature and heterogeneous composition of the immature CD4-8+ subset in normal thymus, it has not been possible to investigate the acquisition of CD4 expression in a clonal population, or to define the functional potential of those CD4-8+ cells that express low levels of CD3/TCR complex. To address these issues, we developed clonal cell lines from thymic lymphomas that represent the immature CD4-8+CD3lo thymocyte subset. In a previous study, we reported that the majority of N-methyl-N-nitrosourea-(MNU) induced thymic lymphomas in AKR mice express a CD4-8+ phenotype (Richie, 1988). These lymphomas also express ThB, a differentiation antigen present on immature but not on mature T-cells (Yotoku et al., 1976), suggesting that they correspond to an immature differentiation stage. The present report describes the phenotypic and functional properties of CD4-8+ MNU-induced lymphomas established in culture as continuous cell lines. The data support the assignment of these lines to the immature CD4-8+ subset and demonstrate their precursor potential. In addition, transcriptional control of CD4 expression and signal transduction via the CD3/TCR complex are assessed prior to and after i.t. transfer.

RESULTS

Phenotypic Analysis of CD4-8+ Lymphoma Cell Lines

Mature thymocytes express either CD4 or CD8 molecules. Recently, however, a minor subset of CD4-8+ thymocytes has been described that displays functional and phenotypic properties characteristic of immature cells (Crispe and Bevan, 1987; Ceredig, 1988; MacDonald et al., 1988; Penit and Vasseur, 1988; Guidos et al., 1989). MNU-induced lymphomas obtained from AKR/J mice generally express a CD4-8+ antigen profile (Richie, 1988). To determine if these lymphomas represent the mature or immature CD8 single-positive (SP) subset, two MNU-induced lymphoma cell lines (829M and 802M) were further characterized. The data in Fig. 1 are consistent with assignment of an immature status for both cell lines since they express high levels of

FIGURE 1. Differentiation antigen phenotype of 829M (upper panel) and 802M (lower panel) lymphoma lines. The dashed line in each histogram shows the profile for cells stained only with an appropriate second-step reagent.
HSA (detected by mAb J11d), undetectable or low levels of CD5 antigen, and low or moderate levels of CD3 and α/β-TCR.

Intrathymic Transfer of CD4⁺8⁻ Cell Lines

Immature CD4⁺8⁻ thymocytes are thought to represent a transient, intermediary subset between the CD4⁺8⁻ and CD4⁺8⁺ developmental stages (Penit and Vasseur, 1988). Previous studies demonstrating the generation of double-positive (DP) cells from immature SP progenitors were carried out with polyclonal normal thymocytes (MacDonald et al., 1988; Nikolic-Zugic and Bevan, 1988; Guidos et al., 1989). To investigate the developmental potential of monoclonal immature CD4⁺8⁻ cells, the 829M and 802M cell lines (Thy1.1⁺) were transferred by i.t. injection into irradiated and syngeneic bone marrow reconstituted AKR/C (Thy1.2⁻) mice. Intrathymic proliferation of lymphoma cells was apparent from the enlarged recipient thymuses and recovery of >90% Thy 1.1⁺ cells. As shown in Fig. 2, the vast majority (93%) of 829M cells recovered after i.t. injection displayed a CD4⁺8⁻ DP phenotype. CD4 expression was also acquired on the 802M cell lines after i.t. transfer, although in this case fewer (65%) of the recovered cells expressed a DP phenotype (data not shown). To investigate the clonal relationship of the injected SP and recovered DP populations, Southern blot analyses of
TCR β-chain gene rearrangements were carried out. Figure 3 shows that the pattern of Jβ2 gene rearrangements in the CD4⁺/8⁻ 829M cells recovered after i.t. transfer was identical to that in the original CD4⁺/8⁻ cell line. These results show that the precursor activity described for heterogeneous populations of normal CD4⁺/8⁻ thymocytes is also a property of the monoclonal 829M and 802M lymphoma lines.

Stable Expression of CD4 on 829M Cells Obtained after Intrathymic Transfer

To determine if the acquisition of CD4 expression was a stable phenomenon, CD4⁺/8⁻ lymphoma cells recovered after i.t. transfer of the 829M line were established in vitro as a cell line designated 7-829M. The clonal pattern of Jβ2 gene rearrangements in this cell line was identical to that found in the parental 829M cells (Fig. 3). Analyses of CD4 expression at various intervals revealed that the 7-829M cells continued to express CD4 during several months of continuous culture (Fig. 4).

Northern Blot Analyses of CD4 Transcripts

CD4 gene expression of 829M cells before and after i.t. transfer was assessed by Northern blot analysis. Cytoplasmic or poly(A⁺) RNA was obtained from the CD4 negative 829M cell line, the CD4 positive 829M lymphoma cells recovered after i.t. injection, and the CD4 positive 7-829M cell line. Figure 5 shows that the 829M cell line did not contain detectable levels of CD4 transcripts. In contrast, the DP cells recovered after i.t. transfer and the 7-829M cell line contained readily detectable CD4 mRNA. These data suggest that the failure of 829M cells to express cell-surface CD4 antigen is not due to masking of the antigen or defective translation, but rather to negative transcriptional regulation or an alteration in CD4 mRNA stability. In either case, exposure of the CD4 negative cell line to the thymic microenvironment positively influences up-regulation of steady-state CD4 transcript levels.

Mobilization of Intracellular Ca²⁺ in Response to CD3/TCR Cross-Linking

Previous studies have shown that anti-CD3 mAb induces a positive but marginal increase in intracellular Ca²⁺ mobilization in the CD4⁺/8⁻ thymocyte subset (Havran et al., 1987). Recent comparative analysis of signaling in normal thymocytes demonstrated that the Ca²⁺ transient induced by anti-α/β-TCR is reduced in comparison with that generated by anti-CD3 (Finkel, Cambier et al., 1989; Finkel, Marrack et al., 1989). It was suggested that this disparity reflects inefficient coupling of CD3 and α/β-TCR in a subpopulation of immature thymocytes. To compare the Ca²⁺ mobilization response induced by CD3 and α/β-TCR mAb and determine if altered signaling via the CD3/TCR complex accompanied conversion to a DP phenotype, the 829M and 7-829M cells were loaded with the Ca²⁺-sensitive indicator Indo-1. As shown in Fig. 6, cross-linked anti-CD3 stimulated a large Ca²⁺ transient that rapidly declined in both cell lines. The Ca²⁺ response stimulated
by cross-linked anti-α/β-TCR was similar in magnitude and kinetics to that induced by anti-CD3 activation, indicating the presence of a functionally coupled receptor complex. After the maximum Ca\textsuperscript{2+} flux was triggered by cross-linked mAb, the level of intracellular Ca\textsuperscript{2+} remained above base line at approximately 0.25 μM for an extended period. Interestingly, anti-α/β-TCR, but not anti-CD3 mAb, stimulated a small but persistent increase in intracellular Ca\textsuperscript{2+} concentration in the absence of cross-linking reagent in the 829M cells.

**DISCUSSION**

Investigations of normal thymocyte subsets involve heterogeneous populations even when the cells are selectively enriched for a particular antigenic profile. The establishment of lymphoma cell lines that express an immature CD4\textsuperscript{8} phenotype provided an opportunity to investigate this unique subset using monoclonal populations. The conclusion that the MNU-induced lymphoma lines represent the immature CD4\textsuperscript{8} subset is based, in part, on their low-level expression of CD5 and high-level expression of J11d antigens. In addition, histological analyses of thymic sections from MNU-treated mice reveal that neoplastic foci develop in the thymic cortex rather than in the medulla, as might be expected if a mature CD4\textsuperscript{8} clone underwent neoplastic expansion (Stettner et al., in press). The most compelling data suggesting that these lymphomas are neoplastic counterparts of the immature CD4\textsuperscript{8} subset are their differentiation potentials. Intrathymic transfer of the 829M and 802M cell lines resulted in acquisition of cell-surface CD4 expression. Since these studies involved monoclonal populations, it was possible to verify, by TCR β-chain gene-rearrangement patterns, that the CD4\textsuperscript{8} cells recovered after i.t. transfer were clonal descendants of CD4\textsuperscript{8} precursors. The 7-829M cell line established after intrathymic transfer remained CD4 positive for several months, indicating that sustained contact with the thymic microenvironment is not required to maintain CD4 expression on these cells.

There is some disagreement regarding CD3 expression on the immature CD4\textsuperscript{8} subset in normal thymus. One study failed to find CD3\textsuperscript{+} cells in the HSA\textsuperscript{+} subset of isolated CD4\textsuperscript{8} normal thymocytes (Shortman et al., 1988). On the other hand, another group reported that failure to express CD3 is not a universal feature of all immature CD4\textsuperscript{8} cells (MacDonald et al., 1988). Rather, their data are consistent with a low frequency of receptor-bearing immature CD4\textsuperscript{8} cells. Low levels of receptor also were detected on 50% of immature CD4\textsuperscript{8} rat thymocytes (Hunig, 1988). Since this study was performed in rats, the data may not be strictly comparable to thymocyte subsets of the mouse. Nevertheless, the results support the notion that the CD3/TCR complex is expressed on a fraction of the immature CD4\textsuperscript{8} subset. Thus, expression of the CD3/TCR complex on the 829M and 802M cell lines is consistent with a phenotypically similar counterpart representing a transitional stage in normal T-cell differentiation.

The CD3 and α/β-TCR molecules expressed on the CD4\textsuperscript{8} 829M and CD4\textsuperscript{8} 7-829M cells appear to be functionally coupled insofar as cross-linked mAb to either component triggered a brisk intracellular Ca\textsuperscript{2+} response. This observation is consistent with the finding that α/β-TCR mAb eliminates a subset of immature thymocytes in fetal thymic organ cultures (Finkel, Marrack et al., 1988). Presumably immature cells that express a functionally coupled CD3/TCR complex undergo negative selection via a clonal deletion mechanism following TCR triggering. If so, one would predict that the 829M and 7-829M cell lines will undergo apoptosis in response to triggering with either cross-linked CD3 or α/β-TCR mAb. Indeed, in transgenic mouse strains
expressing class I restricted TCRs, clonal deletion appears to be operative at or prior to the DP maturation stage (Kisielow et al., 1988 and Sha et al., 1988). Thus, it is likely that cells in the immature CD4*8* compartment are subject to negative (and perhaps positive) selection processes.

Previous studies have not directly addressed the issue of transcriptional or translational control of CD4 expression in immature CD4*8* cells. The Northern blot data in this report demonstrate that CD4 transcripts are not detected in the 829M cell line until conversion to the DP stage after i.t. transfer. Another group suggested that all immature CD4*8* cells express CD4 molecules at a density below the level of detection in standard cytofluorometric assays (Nikolic-Zugic et al., 1989). It is conceivable that the CD4*8* lymphoma lines described in this report constitutively express CD4 transcripts and surface protein at undetectably low levels. If so, the appearance of CD4 after i.t. transfer may be due to up-regulation of expression rather than de novo synthesis. In either case, the present data suggest that failure to express detectable CD4 antigen on the immature CD4*8* subset is regulated at the transcriptional level. Further studies involving nuclear run-on experiments are necessary to determine if the lack (or very low levels) of steady-state CD4 transcripts in CD4*8* cells reflect a block in transcription as opposed to alterations in CD4 mRNA processing or stability.

The MNU-induced lymphoma cell lines appear to be reversibly blocked at the immature CD4*8* differentiation stage. It is not clear why primary MNU-induced lymphomas and derived cell lines fail to express CD4. MNU is cytotoxic and results in profound depletion of the thymic cortex. However, the differentiation-inducing microenvironment of the thymus appears to remain intact since normal cellularity and CD4*8* subset distribution are restored within 2 weeks after treatment (Stettnier et al., in press). Nevertheless, MNU may induce subtle changes in thymic infrastructure that could restrict the developmental program of CD4*8* neoplastic cells, which retain the ability to acquire CD4 expression when removed from the modified thymic microenvironment. Perhaps an MNU-induced mutation generates a unique intrathymic peptide that activates transformed thymocytes via the CD3/TCR complex. Activation, in conjunction with a deficiency in appropriate maturation stimuli, may block the cells at the immature CD4*8* stage of differentiation. Although speculative, this notion is supported by the recent report that TCR cross-linking inhibits CD4 acquisition by the immature CD4*8* normal thymocyte subset (Hunig, 1988). In any event, the cell lines described in this investigation provide useful monoclonal models for further investigations of the immature CD4*8* stage of thymocyte differentiation.

**MATERIALS AND METHODS**

**Establishment of T-Lymphoma Lines**

AKR/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). AKR/C mice were purchased from Cumberland View Farms (Clinton, TN). Thymic lymphomas were induced by a single i.p. injection of 75 mg/kg of MNU into 4–6-week-old AKR/J mice. Cell lines were established in DMEM (Flow Laboratories, McLean, VA) containing 10% fetal calf serum (FCS) (Hy-Clone Laboratories, Logan, UT) and supplemented with 4 x 10⁻⁵ M 2-ME, 4 mM L-glutamine, 1 x nonessential amino acids, and 1 mM sodium pyruvate. Parent cell lines were cloned and subcloned by limiting dilution in 96-well microtiter plates.

**Antibodies**

Hybridomas 53-6.7 (anti-CD8) and 53-7.3 (anti-CD5) were obtained from Dr. J. Ledbetter (Ledbetter and Herzenberg, 1979). Hybridoma GK 1.5 (anti-CD4) (Dialynas et al., 1983) was obtained from Dr. F. W. Fitch. The J11d hybridoma-producing mAb that recognizes an epitope of HSA was obtained from Dr. M. Bevan (Crispe and Bevan, 1987). A hamster mAb, 500A2, specific for the ε chain of murine CD3 was provided by Dr. J.P. Allison (Allison, et al., 1987). MAb H57-157 detecting a framework determinant of the α/β-TCR was obtained from Dr. R. Kubo (Kubo et al., 1989). Second-step reagents included affinity purified, fluorescein isothiocyanate (FITC) conjugated mouse antirat immunoglobulin Ig (Jackson ImmunoResearch, Avondale, PA) and goat antihamster Ig (Cooper Biomedical, Inc., Malvern, PA). FITC conjugated anti-Thy 1.1 (clone TN-26) and anti-Thy 1.2 (clone TS) were
purchased from Sigma Chemical Co. (St. Louis, MO). For two-color staining, FITC conjugated anti-CD8 (clone 53-6.7) and phycoerythrin (PE) conjugated anti-CD4 (clone GK 1.5) were purchased from Becton Dickinson (Mountain View, CA). Primary antibodies were airfuged to remove aggregates.

**Immunofluorescence Staining and Flow Cytometry**

Single- and two-color staining were performed as previously described. Immunofluorescence was analyzed on a FACS IV (Becton-Dickinson, Mountain View, CA) using a 488-nm argon ion laser. Dead cells were excluded from analysis by forward light scatter. Data were collected using a three-decade log amplifier, stored in the list mode of a Consort 40 PDP/11 based computer. For two-color analysis, contour plots were generated with quadrants based on the profile of unstained cells and cells stained with a single mAb.

**Intrathymic Transfers**

Irradiated (1100R) AKR/C mice were reconstituted within 24 hr with 5x10⁶ AKR/C bone marrow cells injected i.v. After anesthesia, the manubrium and one or two sternabrae were incised and retracted laterally to expose the thymus. Ten μl of lymphoma cells (10⁶/ml) were infused with a 27-gauge needle into the most accessible lobe. The skin was closed with 6-0 Surgilene (American Cyanamide Co., Danbury, CT) and the mice were allowed to recover in a warm cage.

**RNA Extraction and Northern Blot Analysis**

Total RNA was isolated as described (Maniatis et al., 1982) from 10⁶ cells. Poly(A)⁺ RNA was isolated using the Fast Track Kit from Invitrogen (San Diego, CA). RNA was electrophoresed in 1.1% agarose containing 2.2 M formaldehyde in morpholinopropanesulfonic acid-acetate-EDTA buffer for 420 volt-hr. After transfer to Magna Nylon (MSI, Westborough, MA), blots were hybridized with a nick-translated probe using Denhardt's reagent and formamide (Wahl et al., 1979), washed (MSI Technical Bulletin 103), and analyzed by autoradiography using Kodak X-OMAT AR film and an intensifying screen at -70°C.

**DNA Extraction and Southern Blot Analysis**

DNA was extracted as previously described (Hoopes and McClure, 1981), digested with Hind III, and electrophoresed on 0.6% or 0.7% horizontal agarose gels at room temperature for 560 volt-hr in Tris-acetate-EDTA buffer. After transfer to Magna Nylon-66 (MSI, Westborough, MA), the blots were prehybridized and hybridized with a nick-translated probe at 65°C in 5xSSPE, 5x Denhardt's reagent, and 0.5% SDS. The blots were washed and analyzed by autoradiography as described before.

**cDNA Probes**

The CD4 probe consists of a 2.4-kb insert of cDNA clone p3C isolated from a thymocyte cDNA library prepared in λgt10 (Littman and Gettner, 1987). The murine Jβ2 probe is a 2.3-kb EcoRI fragment of cosmid clone 2.3 W7 subcloned in pUC8 and hybridizes to the entire Jβ2 gene-segment cluster (Malissen et al., 1984).

**Calcium Measurements**

Cells were loaded with 1 μM indo-1/AM (Molecular Probes, Eugene, OR) in RPMI 1640 (1% FCS) for 30 min at 37°C, gently pelleted and resuspended in an equal volume of HBSS (1% FCS), and kept at room temperature. Fluorescence measurements were obtained on an Alpha Scan fluorometer (Photon Technology Inc., Princeton, NJ) equipped with dual emission detectors. Background fluorescence was determined from an aliquot of unloaded cells processed in parallel with the loaded cells at densities of 0.5-1x10⁶ cells/ml. Prior to data acquisition, cells were warmed to 37°C in the water-jacketed cuvette holder. At the end of each recording, digitonin was added to a final concentration of 100 μM to obtain Rmax and then EGTA/Tris to obtain Rmin. The intracellular free-calcium concentration ([Ca⁺⁺]) was calculated from the 404 nm/490 nm ratio (Grynkieiewicz et al., 1985) except that Rmin and Rmax were modified by a viscosity correction factor of 1.15 (Poenie, 1990).
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