T-Cell-Subset Characterization of Human T-CLL

By Ellis L. Reinherz, Lee M. Nadler, David S. Rosenthal, William C. Moloney, and Stuart F. Schlossman

Circulating peripheral blood tumor cells in four cases of chronic lymphoproliferative disease were immunologically characterized. By the use of T-cell-specific heteroantisera and indirect immunofluorescence, all were shown to involve proliferation of malignant T cells. Three cases demonstrated morphologic and clinical features consistent with chronic lymphocytic leukemia (CLL), and one case presented as a lymphosarcoma cell leukemia. Antisera specific for normal human T-cell subsets defined the malignant T cells in each case as arising from the TH2 subset. This subset normally constitutes approximately 80% of human peripheral blood T cells. Terminal deoxynucleotidyl transferase (TdT) was not detected in any of the T-cell CLL cases, thus supporting the notion that T-cell CLL represents a malignancy of a mature phenotype. The one patient with lymphosarcoma whose tumor cells were TdT-positive subsequently developed T-cell acute lymphoblastic leukemia (ALL). Moreover, la-like antigen (p23,30) was detected on two of these tumor cell populations. In addition, it was shown that not all tumor cells were E-rosette-positive, since only cells from 3 of 4 patients were capable of forming spontaneous rosettes. These findings demonstrate that heteroantisera can provide an additional important tool for dissecting the heterogeneity of T-cell leukemias and for relating them to more differentiated normal T cells.

IN ALMOST all cases chronic lymphocytic leukemia (CLL) is derived from malignant clonal expansion of B cells. Evidence for this has been provided by the identification of monospecific immunoglobulins as well as complement receptors on the CLL cell surface and the demonstration that these cells synthesize immunoglobulin light chains in vitro. However, a small percentage of CLL cases are malignancies of T cells, as defined by T-cell-specific heteroantisera and E-rosetting techniques. This latter group represents less than 5% of cases of CLL and manifests some distinctive clinical signs including skin infiltration, often minimal peripheral lymphadenopathy, and moderate splenomegaly. Morphologically these cells are said, in part, to have unusual lymphoid characteristics such as azurophilic cytoplasmic granules, generous cytoplasm, and convoluted nuclear membranes.

Over the last several years considerable progress has been made in defining phenotypically and functionally distinct subsets of normal human T cells. In previous studies it was shown that peripheral T cells could be divided into two groups on the basis of differential expression of TH2 antigen(s) on their surfaces. Approximately 20%-30% of human peripheral T cells were TH2+.

From the Division of Tumor Immunology, Sidney Farber Cancer Institute, and the Division of Hematology, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.
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Address reprint requests to Ellis L. Reinherz, M.D., Division of Tumor Immunology, Sidney Farber Cancer Institute, 44 Binney Street, Boston, Mass. 02115.
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SUBSET DERIVATION OF T-CLL

whereas 70%-80% were TH₂⁻ with rabbit or equine heteroantisera. Functional studies demonstrated that the TH₂⁺ subset contained the cytotoxic effector and suppressor cell population. In contrast, the TH₂⁻ subset, although numerically representing the majority of circulating peripheral lymphoid cells in man, did not effect either function. Rather, the TH₂⁻ population helped in the generation of cytotoxicity and responded optimally to soluble antigens. Additional techniques using alternative approaches, including autoimmune sera and differential Fc binding for IgG and IgM (Tγ and Tμ), have further supported the notion of phenotypic and functional heterogeneity among human T cells.¹³,¹⁵,¹⁶

Given the existence of human T-cell subsets and the clonal nature of human malignancy, the possibility that human T-cell CLL involved proliferation of individual subsets of human T cells was investigated.¹⁸ This report provides evidence that T-cell CLL represents a proliferation of cells contained within a distinct T-cell subset in man.

MATERIALS AND METHODS

Isolation of Tumor Cells

Human peripheral blood mononuclear cells were isolated from tumor-bearing patients in the leukemic phase of the disease by Ficoll-Hypaque density-gradient centrifugation (Pharmacia Fine Chemicals, Piscataway, N.J.) according to the method of Böyum.¹⁹ The mononuclear population so obtained was more than 90% tumor cells by Wright-Giemsa morphology.

Sheep Red Blood Cell Rosetting

Spontaneous rosette formation with sheep erythrocytes (E rosettes) was performed by methods previously described.²⁰

Analysis of Tumor Cells With Heteroantisera

ATG₁ and ATG₂ represent T-cell-specific equine antihuman thymocyte globulin. These heteroantisera have previously been shown to be reactive with human cells of T-cell lineage but unreactive by immunofluorescence with normal human B cells, null cells, monocytes, lymphoblastoid B-cell lines, or non-T-cell lymphoblastic leukemias.¹⁷ Equine anti-TH₂⁺ antisera reacted with 20% ± 5% of the normal peripheral human T cells of each of 50 normal donors who were tested. Preparation and specificity were the subjects of a prior report.¹⁷ A99 is a rabbit antihuman T-cell heteroantisera prepared by immunizing rabbits with purified plasma membrane fractions from CEM, a human T-cell lymphoblastoid culture line. This antisera was made T-cell-specific by multiple absorptions on human B-cell lines.³¹ Anti-p23,³⁰ preparation and specificity have been defined previously.²²,²³

Equine heteroantisera were reacted with each population of tumor cells and developed with fluorescein-conjugated IgG fraction rabbit antihorse IgG (R/H FITC) (Cappel Laboratories, Downington, Pa.) for analysis on a fluorescence-activated cell sorter (FACS) (Becton-Dickinson, Mountain View, Calif.). In brief, 1–2 × 10⁶ tumor cells were treated with 0.15 ml of equine heteroantisera at a dilution of 1:125, incubated at 4°C for 30 min, and then washed twice. The cells were next reacted with 0.15 ml of a 1:50 dilution of R/H FITC for 30 min, centrifuged, and washed three times. These cells were then analyzed on the FACS at a rate of 500–1000 cells/sec, and the intensity of fluorescence per cell was recorded on a pulse height analyzer.²⁴,²⁵ Background staining was obtained by substituting a 0.15-ml aliquot of 1:125 dilution normal horse IgG for the specific antibody and staining with R/H FITC as described previously. Tumor cell reactivity with the rabbit heteroantisera anti-p23,³⁰ and A99 was determined in an identical fashion except that fluoresceinated F(ab)²₃ goat antirabbit Fc was substituted for R/H FITC and background control was standardized with normal rabbit serum.²³ Analysis of surface immunoglobulin on tumor cells was performed by direct immunofluorescence technique using isotype-specific goat antihuman IgG, IgA, and IgM, which were fluorescein-conjugated (Atlantic Antibodies, Westbrook, Me.) and processed by FACS.
Table 1. Clinical Features of Chronic T-Cell Lymphoproliferative Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (mo)</th>
<th>Clinical Course</th>
<th>Adenopathy</th>
<th>Organomegaly</th>
<th>Bone Marrow Involvement</th>
<th>Leukemia Cutis</th>
<th>CNS Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>55</td>
<td>Cervical, occipital, inguinal, axillary</td>
<td>Hepatomegaly, splenomegaly</td>
<td>+</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>13</td>
<td>None</td>
<td>Hepatomegaly, splenomegaly</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>34</td>
<td>16 + *</td>
<td>Cervical, occipital, epitrochlear, axillary, abdominal</td>
<td>None</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>54</td>
<td>12</td>
<td>Cervical, inguinal, axillary at 3 mo</td>
<td>Hepatomegaly, splenomegaly at 3 mo</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*Still alive.
†Only aspirate performed.

Terminal Deoxynucleotidyl Transferase

Terminal deoxynucleotidyl transferase (TdT) was analyzed by Dr. Ronald McCaffrey employing either a biochemical technique or an immunofluorescence technique or both.26

RESULTS

Clinical Features and Laboratory Findings in Patients With Chronic T-Cell Lymphoproliferative Disorders

Table 1 shows clinical data on each of 4 patients with chronic T-cell lymphoproliferative disease. Ages ranged from 34 to 68 yr in this group. Lymph node enlargement was initially observed in 2 of 4 patients, whereas mediastinal masses were noted in none. Patients 1 and 2 presented with hepatosplenomegaly, and patient 4 developed hepatosplenomegaly 3 mo into her disease. Skin involvement with leukemia cutis was proved by biopsy in 3 of 4 patients. In addition, a striking propensity for central nervous system (CNS) involvement was detected. Three of 4 patients in this group who ultimately died did so as a direct consequence of CNS tumor infiltration. The total length of clinical disease was as short as 1 yr and as prolonged as 4.5 yr.

In Table 2 the relevant clinical laboratory hematologic parameters are listed. White blood cell count ranged from 21,300 to 300,000/cu mm, with 45%–94% lymphoid cells on Wright-Giemsa stain of peripheral blood. Typical morphologic features of the tumor cell populations are seen in Fig. 1. As shown, tumor cells from patient 1 (Fig. 1A) were prolymphocytic in appearance. Patient 2 (Fig. 1B) had a

Table 2. Laboratory Features of Chronic T-Cell Lymphoproliferative Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>WBC on Coombs Test Immuno-</th>
<th>Differential</th>
<th>Hematocrit</th>
<th>(direct/</th>
<th>lgG/lgA/lgM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$89 \times 10^3$</td>
<td>94 lymphs, 6 polys</td>
<td>40.0</td>
<td>-/-</td>
<td>1080/60/114</td>
</tr>
<tr>
<td>2</td>
<td>$300 \times 10^3$</td>
<td>90 lymphs, 10 polys</td>
<td>36</td>
<td>-/-</td>
<td>980/121/86</td>
</tr>
<tr>
<td>3</td>
<td>$21.3 \times 10^3$</td>
<td>33 prolymphs, 12 lymphs, 50 polys, 5 monos</td>
<td>48.7</td>
<td>-/-</td>
<td>N.D.†</td>
</tr>
<tr>
<td>4</td>
<td>$73 \times 10^3$</td>
<td>85 atypical lymphs, 10 lymphs, 3 monos, 2 eos</td>
<td>45</td>
<td>+/-</td>
<td>1480/324/120</td>
</tr>
</tbody>
</table>

*Expressed as milligrams per deciliter.
†N.D. = not done.
Fig. 1. Leukemic cells in peripheral blood from (A) patient 1, (B) patient 2, and (C) patient 3.
tumor population with a nuclear pattern that was strikingly cerebriform. Patient 3 (Fig. 1C) had tumor cells with lymphosarcomatous morphology. Not shown are tumor cells from patient 4, which were similar to those from patient 2 in the convoluted appearance of the nuclear pattern. Patients in this group were not anemic, despite the high peripheral white blood cell count and marrow involvement and/or virtual replacement in 3 of 4 patients studied. Patient 4 had red blood cells that were positive in a direct Coombs test. This patient subsequently developed a significant hemolytic episode during her disease (data not shown). Immuno-electrophoresis revealed no M component or diminution in normal isotype levels in those patients studied at the initiation or during clinical disease.

**Immunologic Characterization of Tumor Cells**

Phenotypic characterization of tumor cell populations is presented in Table 3. Tumor cells in 3 of 4 patients bore detectable sheep erythrocyte rosette receptors. Within these three E* populations more than 90% of tumor cells were E-rosette-positive (data not shown). Moreover, antisera directed against human

### Table 3. Immunologic Characterization of T-Cells in T-Cell Chronic Lymphoproliferative Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>ATG1</th>
<th>ATG2</th>
<th>A99</th>
<th>Anti-TH2*</th>
<th>E-Rosette Capacity</th>
<th>Anti-p23,30</th>
<th>Surface Ig</th>
<th>Cap</th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
<th>TdT</th>
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<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>4</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Fig. 2. FACS histogram showing the fluorescence profiles of peripheral human T cells (A) and leukemic T cells from 1 of 4 patients (B) when stained with ATG2 and R/H FITC (solid lines). Normal T cells can be divided into two subsets: one weakly reactive (TH2−) and the other strongly reactive (TH2+) on FACS (2A). As shown, the leukemic population derives from the TH2− population (2B). Background fluorescence staining (dotted lines) was obtained by incubating cells with normal horse IgG and developing with R/H FITC.
immunoglobulin isotypes IgG, IgM, and IgA were unreactive with all the tumor populations by indirect immunofluorescence. In contrast, heteroantisera specific for the human T cell, produced in both horse (ATG₁ and ATG₂) and rabbit (A99), were reactive with all four tumor cell populations.

A fluorescence histogram of normal human peripheral blood T cells analyzed on FACS with ATG₂ by indirect immunofluorescence is typically bimodal (Fig. 2A). Approximately 80% of peripheral blood T cells react with low-intensity fluorescence and are TH₂⁻, whereas 20% are highly fluorescent and are TH₂⁺. Despite the variability in morphology, all 4 patients' tumor cells were TH₂⁻ (Fig. 2B). In addition, by using the specific anti-TH₂⁺ antibody that normally defines 20% of peripheral human T cells (Fig. 3A), it was shown that no tumor cells from any of these individuals were reactive. Figure 3B demonstrates the absence of reactive tumor cells from patient 2, but this pattern was similar in all 4 patients.

In addition, we investigated tumor cell surface expression of Ia antigen and intracellular TdT activity. Tumor cells from patients 1 and 4 were Ia-positive with the use of anti-p23,30 and indirect immunofluorescence. When TdT activity was determined for each human population, only patient 3 was found to be TdT-positive. Approximately 8 mo following diagnosis this individual developed T-cell acute lymphoblastic leukemia (T-ALL), with a white blood cell count greater than 100,000/cu mm containing approximately 80% blasts.

**DISCUSSION**

In the present study, circulating peripheral blood tumor cells in 4 patients with lymphoproliferative disease were analyzed. The significant reactivity of both rabbit
and equine antihuman T-cell heteroantisera in each case defined these cells as
being of T-cell lineage. Three patients demonstrated features characteristic of
T-cell CLL, whereas 1 patient was best classified as having T-cell lymphosar-
coma/leukemia. All 4 cases represented malignant proliferation of a specific T-cell
subset, namely the TH2− subset in man. This was shown with the use of highly
selective heteroantisera. In no case was surface IgG, IgM, or IgA detectable using
isotype-specific antisera. In 3 of 4 patients, tumor cells formed spontaneous rosettes
with sheep erythrocytes. However, 1 patient’s tumor cells, although clearly reactive
with T-cell-specific antisera, did not form rosettes with sheep erythrocytes. This
latter finding suggests that multiple modalities for tumor cell analysis are impor-
tant in defining the precise nature of tumor cell origin.

To date, approximately two dozen cases of documented T-cell CLL have been
reported. The morphologic appearance of these cells is variable. Yodoi et al.10
reported 2 cases of T-cell CLL detected with heteroantisera and E-rosetting
technique. One patient had tumor cells that appeared morphologically identical
with those of the common variety of B-cell CLL, with scant cytoplasm and clumped
nuclear chromatin. Tumor cells from their second patient were lymphosarcomatous
in appearance, with “larger immature cell characteristics.” Catovsky et al.7
reported 4 cases of prolymphocytic leukemia, one of which was E+, EAC−, sIg−,
whereas the remainder were E−, EAC+, sIg+. Despite the T-cell lineage of cells
from only 1 of these patients, the morphologic appearances were indistinguishable
among all tumor cell populations in this group. Subsequently, Brouet et al.11
documented 11 cases of T-cell CLL: 2 prolymphocytic, 6 lymphocytic with
azuriphilic cytoplasmic granules, and 3 of mature lymphoid type. In the present
study we describe 3 cases of T-CLL. The morphology of the nuclear pattern of
tumor cells from 2 of these individuals was cerebriform. A third patient had
lymphoid tumor cells of a prolymphocytic type. Moreover, the cells from the patient
destined to develop an acute leukemia appeared lymphosarcomatous. Despite their
morphologic variability, these tumor cells all represent malignant transforma-
tions of the TH2− T-cell subset.

Clinical presentation in this group of patients was also variable, since 2 of 4
patients had peripheral lymph node enlargement at presentation, and only 1 of
these had hepatosplenomegaly. None of the patients had a mediastinal mass, an
associated finding in acute T-ALL and lymphoblastic lymphoma of childhood.27
Perhaps the lack of thymic mass is related to the maturity of T cells and/or the
nature of the T-cell subset.

Bone marrow involvement was proved by biopsy in 3 of 4 patients at the time of
presentation. Patient 4, who clinically represents CLL, had a negative bone marrow
aspirate (< 5% lymphoid cells); however, no biopsy of bone marrow was obtained
in this patient. Nevertheless, despite considerable marrow infiltration and white
blood cell counts greater than 70,000/cu mm in 3 of 4 patients, the hematocrits
were essentially normal. Since T cells have been shown to produce a factor or
factors that facilitate induction of erythroid differentiation from the stem cell
compartment in vitro, it is conceivable that the absence of anemia could be due, in
part, to production of such a helper factor by malignant T cells.28

The evolution of leukemia cutis and leukemic meningitis in 3 of 4 patients is an
unusual feature of the T-cell malignancies reported herein. Both normal and
malignant T cells have been noted to have striking migratory patterns into CNS,
skin, and testes. In this regard it is interesting to note that the TH$_2^-$ cell, like the Ly1 T cell that has been shown to be involved in delayed hypersensitivity in the mouse, has a capacity for effector functions that involve migration into skin.

In the present study we could not demonstrate TH$_2^+$ T-CLL cells. Since the TH$_2^+$ subset represents only 20% of the peripheral T-cell population, it is possible that we have not examined a large enough series of T-CLL cases, and that such cases exist. This assumes an equal susceptibility of TH$_2^-$ and TH$_2^+$ cells for a “transforming event,” as appears to be the case among B cells with differing surface isotypes in multiple myeloma. A recent report demonstrating the presence of a T-cell CLL that suppressed autologous and allogeneic BFU-E suggests that this particular T-CLL might be of such a TH$_2^+$ subset, since the normal TH$_2^+$ subset is suppressive in a variety of T, T- and B-cell interactions. In the murine system, lymphomas and leukemias of all the mature Ly alloantigen phenotypes have been described. For example, mouse T-cell tumors are phenotyped as either Ly1 (helper cell phenotypes) or Ly2,3$^+$ (suppressor/cytotoxic cell phenotypes), but rarely both. This restricted expression of Ly antigens on either 1-ethyl-1-nitrosourea-induced or spontaneously occurring AKR lymphomas suggests that the target for leukemogenesis can occur in a partially differentiated cell expressing, or destined to express, a mature T-cell phenotype. Taken together, these data imply obvious similarities between murine and human lymphoproliferative disorders.

Additional evidence pointing toward the heterogeneity of T-cell malignancies found in man is the demonstration that tumor cells from 2 of 4 patients were shown to be p23,30-(Ia)-positive. This membrane glycoprotein antigen complex of 23,000-d and 30,000-d subunits was initially shown to be specific for B cells and 20% of human null cells but unreactive with normal human T cells. Moreover, it was reactive with 85% of childhood ALL and all acute myelogenous leukemias. Subsequently it has been demonstrated that normal human T cells, after alloantigen or mitogen activation, express p23,30. This study documents that both Ia-positive and Ia-negative T-cell CLLs exist in man. Whether the Ia positivity is a reflection of the activation state of these cells or the phenotypic expression of a rare fraction of these T cells remains to be determined.

The human TH$_2^-$ subset contains a population of T lymphocytes that provide a variety of helper functions. In this regard it is noteworthy that the 1 patient who developed a Coombs-positive hemolytic anemia had TH$_2^-$ tumor cells that were Ia-positive. It is possible that these cells provided help to induce autologous B cells to produce antibody with anti-RBC specificity. Moreover, other patients with chronic T-cell lymphoproliferative disease, especially Sézary syndrome, develop Coombs-positive hemolytic anemias.

Previous studies have indicated that TdT activity reflects the degree of cell maturity. Thus a small percentage of murine marrow cells and a significant number of thymocytes possess this enzyme activity, whereas peripheral nodes and spleen cells do not. TdT activity is detectable in the vast majority of cases of ALL. The absence of TdT in the 3 cases of T-CLL provides yet another argument for the mature state of differentiation of these human cells. Furthermore, the findings in the single patient with detectable TdT activity in his tumor population, who subsequently developed unequivocal ALL, support this view.

Previous work has shown that the programming of specific cell function appears
to be linked to the expression of a particular cell surface phenotype. Therefore, the functional repertoire of the human TH2 cell is distinct from that of the TH1 cell. Since human T-cell proliferative disorders can now be identified as arising from distinct T-cell subsets, any functional properties of these tumor cells might be characteristic of the specific repertoire possessed by that individual subset. Yet to be determined is what percentage of T-cell lymphoproliferative disorders retain functional properties. However, it has been shown that some T-cell malignancies can help (whereas others suppress) immunoglobulin production by normal B cells. This again supports the notion that functional as well as phenotypic heterogeneity will become apparent during the investigation of human T-cell disorders.

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