A patient with T-cell chronic lymphocytic leukemia (T-CLL) is reported whose cells demonstrate in vitro suppression of normal lymphocyte mitogen stimulation. The patient, who remains in Rai's clinical stage 0 on no therapy after more than 24 mo of observation, has shown a less aggressive clinical course than is usually attributed to T-CLL. His peripheral blood lymphocytes (PBL) were characterized by functional assays as well as surface markers. Over 90% of the patient's PBL formed rosettes with sheep erythrocytes and were lysed by two T-cell-specific antisera plus complement, while less than 1% bore surface immunoglobulins, and only 3% had complement receptors. In addition, 45% of the PBL demonstrated Ia-like antigens, more than 50% expressed a receptor for the Fc portion of IgG (Tγ), and most of the sheep erythrocyte rosettes were inhibited by theophylline. The patient's cells failed to respond to several mitogens and they caused marked suppression of lymphoproliferative responses to normal PBL to phytohemagglutinin (PHA) and concanavalin A (Con-A). The patient's lymphocytes also exhibited antibody-dependent cytotoxic activity (ADCC) against antibody-coated nucleated target cells, but lacked demonstrable natural killer (NK) activity. This patient's T-CLL cells appear to represent the clonal expansion of a subset of T cells with a previously undescribed pattern of suppressor and cytotoxic activities.

Characterization of a Suppressor T-Cell Chronic Lymphocytic Leukemia With ADCC But Not NK Activity

By Franco Pandolfi, Douglas M. Strong, Robert B. Slease, Michael L. Smith, John R. Ortaldo, and Ronald B. Herberman

A patient with T-cell chronic lymphocytic leukemia (T-CLL) is reported whose cells demonstrate in vitro suppression of normal lymphocyte mitogen stimulation. The patient, who remains in Rai's clinical stage 0 on no therapy after more than 24 mo of observation, has shown a less aggressive clinical course than is usually attributed to T-CLL. His peripheral blood lymphocytes (PBL) were characterized by functional assays as well as surface markers. Over 90% of the patient's PBL formed rosettes with sheep erythrocytes and were lysed by two T-cell-specific antisera plus complement, while less than 1% bore surface immunoglobulins, and only 3% had complement receptors. In addition, 45% of the PBL demonstrated Ia-like antigens, more than 50% expressed a receptor for the Fc portion of IgG (Tγ), and most of the sheep erythrocyte rosettes were inhibited by theophylline. The patient's cells failed to respond to several mitogens and they caused marked suppression of lymphoproliferative responses to normal PBL to phytohemagglutinin (PHA) and concanavalin A (Con-A). The patient's lymphocytes also exhibited antibody-dependent cytotoxic activity (ADCC) against antibody-coated nucleated target cells, but lacked demonstrable natural killer (NK) activity. This patient's T-CLL cells appear to represent the clonal expansion of a subset of T cells with a previously undescribed pattern of suppressor and cytotoxic activities.

CASE REPORT

M.B. is an asymptomatic, previously healthy 41-yr-old white male who, in March 1978, was found to have an elevated white cell count. Detailed history revealed no obvious etiologic factors, and physical examination was entirely normal. Complete blood count showed a hemoglobin of 13.2 g/dl, hematocrit 39%, WBC 30,600/cu mm with 5% segmented forms and 95% lymphocytes, and a platelet count of 395,000/cu mm. The peripheral blood smear evaluation demonstrated a predominance of small lymphocytes, with small to moderate amounts of cytoplasm, densely clumped nuclear chromatin without convolutions or cleaving, and rare nucleoli. Bone marrow aspiration and biopsy specimens revealed increased numbers of small lymphocytes, comprising approximately 30% of nucleated elements. Photomicrographs of the patient's lymphocytes as seen by light and transmission electron microscopy are shown in Fig. 1. Cytochemical stains of the peripheral blood and bone marrow lymphocytes showed granular 8-glucuronidase and acid-phosphatase positivity in nearly all the cells, the latter enzyme sensitive to tetratract. Scanty periodic acid Schiff (PAS) positive material was found in a few cells, but most were negative. Myeloperoxidase and nonspecific esterase activities were both negative. Chest x-ray and routine blood chemistries, including serum protein elec-
tropheresis and quantitative immunoglobulins, were normal. Delayed cutaneous hypersensitivity reactions to \textit{Candida} and SK-SD antigens were also normal. On the basis of the peripheral blood and bone marrow lymphocytosis, the clinical diagnosis of CLL, Rai’s stage 0, was made.

Since initial presentation, the patient has remained entirely asymptomatic and physical examination continues to be normal. Total peripheral lymphocyte counts have fluctuated from 20,000 to 50,000/cu mm.

**MATERIALS AND METHODS**

**Surface Markers**

Isolation of peripheral blood lymphocytes (PBL) from heparinized venous blood was performed by Ficoll-Hypaque density gradient centrifugation, and the determination of percentage of lymphocytes forming conventional rosettes with sheep erythrocytes (sheep rosette-forming cells—SRFC) were performed as previously described. Stable rosettes were evaluated in the same manner with the exception that after the centrifugation, the pellet was incubated for 1 hr at 37°C instead of at +4°C prior to reading.

The effect of theophylline on the frequency of SRFC was studied using the technique described by Limatibul et al. with few modifications. PBL were suspended at $5 \times 10^6/$ml in a solution of theophylline (Calbiochemical, San Diego, Calif.) 0.01 M in HBSS. The cells were incubated at 37°C for 2 hr, and 50A of this suspension was then mixed with 50A of SRBC and tested for rosetting as in the conventional assay. Cells that lacked the ability to form rosettes after preincubation with theophylline were defined as theophylline-sensitive (T-sens).

Assays to determine the frequency of cells with Fc-IgM receptors (Tµ) and Fc-IgG receptors (Tγ) were performed as described by Moretta et al. and Ferrarini et al. EAC-RFC assays were performed using both mouse (mo) and human (hu) complement, and human IgM-coated sheep erythrocytes using a method previously described. To determine the frequency of SmIg-bearing lymphocytes, cells were labeled with fluoresceinated, pepsin-digested, affinity-chromatography-purified preparations of goat anti-human IgM, IgD, IgA, IgG, and Fab (to detect total Ig). Labeled cells were then analyzed using the fluorescence-activated cell sorter (FACS) as previously described.

Desoxynucleotidyl transferase (TdT) activity on the patient’s peripheral blood and bone marrow mononuclear cells was kindly performed by Dr. Fred Bollum, using a previously described immunofluorescence method.

**Studies With Heteroantisera**

The cytotoxicity test was performed as described by Amos et al. The results are expressed as a cytotoxicity index (CI), i.e., the ratio between cells killed in the test with specific antisera at a predetermined dilution (AS) and the control with complement (C') alone, calculated by the following formula:
IMMUNOLOGIC CHARACTERIZATION OF A T-CLL

Five different antisera were used: (1) Anti-CTC—raised in rabbits by immunization with human cultured T-cells (CTC). These CTC were grown in the presence of conditioned medium (CM) from phytohemagglutinin (PHA) stimulated normal PBL as described by Morgan et al. and Ruscetti et al. and possess T-cell surface markers. The antiserum was absorbed twice at 1:1 packed cells/serum ratio at 37°C for 30 min with an Epstein-Barr virus (EBV) transformed B-cell line derived from the same donor of the CTC used for immunization. It reacted by cytotoxicity with PBL T cells but not with B cells or monocytes, and reacted specifically with leukemia cells or cell lines in the T-cell lineage (Maca et al., and Pandolfi et al., submitted). (2) Anti-HTY—prepared as described by Woody et al. and rendered T-cell-specific with multiple absorptions on B-cell lines (Pandolfi et al., submitted). This antiserum recognizes antigens on peripheral blood T lymphocytes, thymocytes, and MOLT-4, a line derived from T-ALL cells. It does not react with normal B cells, B-CLL cells, or monocytes. (3) Anti-HTY,—anti-HTY was further absorbed with PBL pooled from 5 healthy donors at 2:1 serum/packed cells ratio. The absorptions were carried out each time for 30 min at room temperature until the derived antiserum (anti-HTY, ) no longer reacted with the PBL of different donors in the cytotoxicity tests. This serum was found to react with thymocytes and MOLT-4 cells, but not with peripheral blood T cells. (4) Anti-p23,30—a gift of Dr. R. E. Humphreys that recognizes Ia-like antigens on human cells. Details on the preparation of this serum, which reacts with thymocytes and MOLT-4 cells, but not with peripheral blood T cells. (5) Abs. AN6-M1-KC1 serum—a gift of Drs. T. N. LeBien and J. H. Kersey. Details on the preparation of this serum, which reacts specifically with a leukemia-associated antigen expressed on non-T, non-B acute lymphoblastic leukemia (ALL) and on chronic myelogenous leukemia (CML) in lymphoblast crisis (LBC), are described elsewhere.

Functional Studies

Responses of lymphocytes to the lectins phytohemagglutinin-P (PHA) (DIFCO Lab., Detroit, Mich.), concanavalin A (Con-A) (Calbiochemical, San Diego, Calif.), a pokeweed mitogen (PWM) (GIBCO, Grand Island, N.Y.) were performed in triplicate as previously described. The results are expressed as a ratio between the stimulated and the control cultures (stimulation index—SI). Mixed leukocyte culture (MLC) reactivities were measured in triplicate as described before. The assay for tetanus toxoid (t.tox) induced blastogenesis utilized 500 Lf/ml, a gift from Dr. F. J. McCarthy (Wyeth Lab., Marietta, Pa.), at optimal concentrations. The assay technique was identical to that of the mitogens, except that t.tox was added and the cultures were incubated for 5 days.

Suppression was determined on lectins and MLC responses by the effect of 10⁶ normal PBL or T-CLL cells on the responses of 10⁶ normal cells in vitro. Percent inhibition is reported as follows:

\[
\text{% Inhibition} = 100 - \left( \frac{\text{SI of normal PBL + T-CLL cells}}{\text{SI of normal PBL}} \right) \times 100
\]

Tests for ADCC and NK activity were also performed with Ficoll-Hypaque-separated cells and effectors. Cells used for targets were (1) K562, (2) MOLT, (3) ALAB, and (4) Chang liver cells. All targets were washed twice in saline and resuspended in complete medium containing 10% FCS. Chang cells were coated for ADCC targets with a rabbit anti-Chang serum. The ⁵¹Cr-release assay was performed as previously described. All reactions were set up in triplicate. Autologous controls (unlabeled target cells added to the labeled targets) were employed to determine baseline release values in all experiments. The percentage of isotope released was calculated by the following formula:

\[
\text{% Release} = \frac{\text{cpm Released from cells during incubation}}{\text{Total cpm incorporated into cells}} \times 100
\]

The percent specific cytotoxicity (A) was calculated as \(A = B - C\), where \(B\) was the percent release in the experimental group, and \(C\) was the percent release in the autologous control. Activity of effector cell cultures was expressed as lytic units (LU). This was calculated as previously described. 1 LU in an effector cell population was defined as the number of cells required to produce 30% specific cytotoxicity. Total lytic units (TLU) were calculated based on the total viable cell recovery in the reactions as a function of lytic activity (total viable cell number divided by the number of cells per LU).

RESULTS

The results obtained in the study of surface markers are shown in Table 1. The great majority of the patient’s peripheral blood mononuclear cells formed rosettes with sheep erythrocytes. The ability of cells to form rosettes at 37°C (stable rosettes—S-SRFC) with sheep erythrocytes has been shown to be a property of T-cell acute lymphoblastic leukemia blasts and normal thymocytes, but not of normal T-PBL. We tested the patient’s cells for this property and found that his cells did not form rosettes at 37°C. Sensitivity of rosette-forming lymphocytes to theophylline has been reported to identify a subset of T cells that can suppress the generation of plaque-forming cells. Rosettes formed with the patient’s cells were inhibited up to 90% by the preincubation with theophylline (T-sens RFC). In addition, over 50% of the T cells bore the receptor for the Fe portion of IgG (Tγ), while only 10% demonstrated the receptor for the Fc portion of IgM (Tμ). EAC-RFC, using either mouse or human complement that identify the receptor for C3d and C3b, respectively, were formed on 2% and 3% of the patient’s lymphocytes. The frequencies of sIgM, −D, −G, and −A-bearing cells were <1%, as determined by FACS analysis. Figure 2 is a FACS fluores-

| Table 1. Surface Markers on the Patient's Peripheral Blood Mononuclear Cells |
|---------------------------------|-----------------|-----------------|
|                                | Percent Positive Cells | Normal Range for PBL (%) |
| SRFC                            | 93               | 60–80           |
| S-SRFC                          | 0                | <1              |
| T-sens RFC                      | 90*              | 10–30*          |
| Tμ cells                        | 10*              | 50–70*          |
| Tγ cells                        | 57*              | 8–15*           |
| SmIg                            | <1               | 5–15            |
| EACmedian-RFC                   | 2                | 8–15            |
| EACmed-RFC                      | 3                | 10–25           |
| TdT                             | <1               | <1              |

* These percentages are expressed as percentages of total SRFC.
cence profile histogram, with increasing fluorescence on the X-axis and the relative number of cells within each fluorescence channel on the Y-axis. The solid-line curve represents unlabeled cells from the patient, M.B., while his anti-Fab-labeled lymphocytes are shown by the dotted-line curve, which is virtually superimposed on the profile of unlabeled cells. For comparison, the fluorescence profile of anti-Fab-labeled cells from a patient with B-CLL, analyzed at the same FACS sensitivity setting, is represented by the dot-dash lines. Fewer than 1% of the T-CLL patient's peripheral blood mononuclear cells demonstrated TdT activity (Table I).

The reactivity with heteroantisera (Table 2) shows that the cells reacted with two specific anti-T sera, anti-HTY and anti-CTC, but expressed neither thymus-leukemia-associated antigens nor the non-T, non-B ALL antigen(s) detected by anti-HTY; and abs. AN6-M1-KC1 sera, respectively. However, 45% of the cells reacted with the anti-p23,30 (Ia-like) serum.

The results of tests of reactivity in assays of lymphoproliferative responses are summarized in Table 3.

The patient's PBL were unable to respond to lectins, t.tox, and MLC as shown by the very low SI obtained in these tests. Furthermore, these cells were unable to stimulate normal PBL in MLC (data not shown).

As both Tγ and T-sens cells were proved by other authors to exert suppressor activity on cell differentiation, the possibility that the lack of reactivity in the lymphoproliferative assays could involve suppressor mechanisms was investigated. When \(10^4\) T-CLL cells were added to \(10^5\) normal PBL, the responses of normal cells to lectins were markedly inhibited, while normal responses in MLC were not effec. Control experiments were performed adding \(10^4\) normal PBL to the responding cells and did not show significant variations.

Because of the finding that these T-CLL cells express receptors for the Fc portion of IgG, the cytotoxicity against antibody-coated targets and targets susceptible to natural killing (NK) was tested. As shown in Table 4, high levels of ADCC were seen. In contrast, no detectable NK cytotoxicity was detected using several NK-susceptible targets.

## DISCUSSION

This report describes a new case of T-CLL. The identification of the patient's mononuclear cells as T lymphocytes was based on their surface properties: lack of B-cell markers (SmIg, C3b and d receptors) and presence of classical T-cell markers, i.e., reactivity
with T-specific heteroantisera and ability to rosette with sheep erythrocytes. This case presents several clinical and immunologic peculiarities: (A) an unusually mild clinical course; (B) a high proportion of Tγ cells and concomitantly a very high percentage of SRFC inhibited by preincubation with theophylline (T-sens); (C) unresponsiveness to mitogens but ability to suppress mitogen-induced proliferative responses of normal lymphocytes; (D) the presence of p23,30 (Ia-like) antigens but lack of early T-cell differentiation markers; and (E) effector activity in ADCC but not NK assays.

Since fewer than 50 cases of T-CLL have been described, the natural history of the illness remains incompletely defined. However, two recent series from Europe and Japan have reported the clinical features of 11 and 16 cases, respectively. Some of these, as well as others, were described in a review by Tobin et al. Patients were generally middle-aged, with widely variable peripheral blood and marrow lymphocytosis. Lymphocyte morphology was sometimes characterized by nuclear convolutions similar to those of cells from patients with Sézary's syndrome. Skin involvement seems to be distinctly more common in T-CLL than in the B-cell variety. Hepatosplenomegaly was also common in both European and Japanese series, but lymphadenopathy, while seen in 15 of 16 Japanese cases, was found in only 1 of the 11 European patients. Clinical courses were generally more aggressive than B-CLL, with only 2 patients surviving over 5 yr.

Unlike other reported cases of T-CLL, our patient presented and has remained in Rai's stage 0, with no lymphadenopathy, organomegaly, skin lesions, anemia, or thrombocytopenia. Though this is a relatively common presenting stage for B-CLL, its uniqueness in the T-CLL variant necessitates careful analysis and review of the diagnostic criteria used in this case. The diagnosis was made on the basis of a persistent and significant peripheral blood and bone marrow lymphocytosis, consisting almost exclusively of mature-appearing T-cells, in the absence of any demonstrable etiologic agent. We are unaware of any reports of lymphoid leukemoid reactions in adults that have persisted for over 24 mo without an obvious causative illness. In addition, the diagnosis was strengthened by the data presented here showing that the patient's PBL primarily represent a single T-cell subset. However, it must be emphasized that monoclonality has not been demonstrated in this case, and that, therefore, we cannot unequivocally confirm the malignant diagnosis.

Assays for detection of receptors for the Fc portion of IgG and IgM (Tγ and Tμ) on human lymphocytes have provided valuable new tools for the identification of subpopulations of T lymphocytes with different functional activities. It has been demonstrated that Tγ cells exert a suppressor activity on B-cell differentiation produced by PWM, while Tμ cells provide help. Among normal PBL, Tγ usually represent about 10% of the T cells, while Tμ comprise more than 50%. Moreover, it has been recently demonstrated that the property of theophylline to inhibit the rosetting of some lymphocytes with sheep erythrocytes identified a subpopulation of T cells (T-sens RFC) that showed suppressor activity on the generation of specific IgM plaque-forming cells. There seems to be a major overlap between Tγ and T-sens cells. Few data are available on the expression of Tμ, Tγ, and T-sens cells in T-CLL. Brouet et al. reported that Tμ were found in five cases of T-CLL, while Tγ were present in a single case. Our patient's cells demonstrated an extraordinarily high proportion of Tγ and T-sens cells and, therefore, appeared to represent the expansion of a subset of T cells whose normal counterpart has suppressor activity. Although there may be some evidence for Tγ cells being in some way related to monocyes, the morphological and cytochemical evidence, as well as nonadherence to nylon wool, suggests that this patient's cells are of lymphocyte lineage.

Several assays were performed to determine if the T-CLL cells retained some functional capabilities. Not surprisingly, the patient's PBL failed to respond to lectins, t.tox, and in MLC, data that are similar to that found in the majority of patients in Brouet's series. However, PBL from our patient strongly suppressed the response of normal PBL to mitogens, even when added at a 1:10 T-CLL/normal PBL ratio. This concentration of T-CLL cells is too low to have an overcrowding effect on the responding cells, and, in fact, replacing T-CLL cells with normal PBL in control experiments did not affect the responsiveness of the cultures.

The T-CLL cells failed to either suppress or stimulate in MLC, despite their suppression of normal PBL mitogen responses and their Ia positivity. Lack of stimulation may be related to lower Ia density than normal B cells, while lack of suppression may be due to a requirement for histocompatibility (HLA-DR) between the suppressor and responder cells. We also assayed for lectin-mediated cytotoxicity (data not shown), since mitogen suppression might be explained on that basis. However, very low activity was found, thus ruling out that mechanism. Also, these cells did not suppress in the pokeweed-mitogen-induced immunoglobulin biosynthesis assay (Drs. T. Waldman and T. Fleisher, personal communication), despite suppression in mitogen proliferation assays. The fact that
the patient retains normal serum immunoglobulin levels supports these data. These cells may therefore be restricted in their activity towards another T-cell subset(s) not involved in B-cell help. Few other cases of T-CLL with suppressor activity have been described in detail. Hoffman et al.26 demonstrated suppression of normal erythroid colony formation by cells from a T-CLL patient, while Uchiiyama et al.25 reported suppression of PWM-induced normal B-cell differentiation by cells from three of six Japanese patients with adult T-cell leukemia.

To assess the state of differentiation of the T-CLL cells, a number of techniques were employed. The patient’s cells were unable to rosette at 37°C with sheep erythrocytes (stable rosettes or S-SRFC). This ability of T cells to form S-SRFC has been detected on thymocytes,50,51 T-ALL,30 the MOLT-3 cell line52 and on mitogen-activated T lymphocytes.56 TdT activity, which is found in most thymocytes and ALL cells but not in normal peripheral blood lymphocytes53 and is thought to represent a marker of early T-cell differentiation, was also undetectable in our case. The patient’s cells did not express detectable early differentiation, as shown by the nonreactivity with anti- HTY, and abs. AN6-M1-KC1 sera. These sera are analogous to other antisera recognizing antigen(s) expressed on T-ALL (and shared with MOLT-4 cell line and thymocytes)50,58 and on non-T, non-B ALL,59,60 respectively.

A significant proportion of these T-CLL cells were Ia positive. T-cell malignancies have been shown to be heterogeneous with regard to the expression of p23,30 (Ia) antigen.18,27 This antigen was considered to be specific for B cells and a subset of human null cells, but was also shown to be expressed on the majority of childhood ALL and on acute myelogenous leukemias.42 More recently, it has been demonstrated that normal human lymphocytes, after mitogen or alloantigen activation, express p23,30 or Ia antigens.61,62 In addition, a major proportion of T-sens cells, i.e., the T-cell subset, with suppressor activity was also shown to express Ia-like determinants.63 Our data confirm at least partial overlap of these two markers on the cells of our patient with T-CLL. The finding of only 45% cytotoxicity with the anti-p23,30 serum may reflect variation in surface density of this antigen among the T-CLL cells, since a higher proportion of these cells were lysed by a higher serum concentration (data not shown), as we have seen with CTC that also express Ia antigens.62 Experiments using monoclonal anti-Ia reagents and the FACS support this concept (data not shown).

We conclude from these data that T-CLL results from the malignant proliferation of a subpopulation of relatively mature T lymphocytes. Other studies of patients with T-CLL also indicated that the leukemia cells were derived from a particular subpopulation of T cells.18,27 In one such report,27 four cases of T-CLL were unreactive with anti-THs serum. This serum was rendered T-cell subset-specific by absorptions on cells of a T-CLL patient and recognized a subset of normal T-PBL with cytotoxic effector and suppressor activity.25 We used the same approach to absorb a specific anti-human T-cell serum with our patient’s cells, and we obtained an antiserum that recognized the theophylline-resistant subset of normal T-PBL (Pandolfi et al., Clin Exp Immunol—In press64). Monoclonal antibodies recognizing these T-cell subsets may provide a more practical approach to characterization of cell populations.65

T cells with Fc receptors for IgG and low affinity receptors for SRBC have been shown to account for the majority of the effector cells mediating ADCC against tumor target cells.66 The strong reactivity in ADCC by the Tγ-CLL cells of our patient indicates that the leukemic cells have retained this characteristic functional capability as well as suppressor activity. In contrast, the cells had no detectable NK activity. This is rather surprising, since NK cells have been found in the same subpopulation as ADCC effector cells,65 and recent studies have even indicated that the same cells may mediate both activities.68 At least three explanations may be offered for the intriguing dissociation of ADCC and NK activities in this patient. First, although the leukemic cells retained their Fc receptors, they may have lost the recognition structures for natural cytotoxicity. Alternatively, the clonal population of Tγ cells may have NK activity but have receptors specific for target cells different from those studied here. Our laboratory69 and others70,71 have obtained evidence that human NK cells are heterogeneous and recognize multiple specificities on target cells. Testing of the patient’s PBL against a broader array of target cells might reveal previously undetected NK activity. Finally, it is conceivable that this patient’s T-CLL cells could represent the clonal expansion of a very small subpopulation of T cells, which, in their normal state, express ADCC but not NK activity. In any case, the dissociation of ADCC from NK activity in our patient offers an excellent opportunity to study in detail the relationship between effector cells for NK and ADCC and also the possible relationship between these cytotoxic functions and suppressor activity.

The characterization of subpopulations of T lymphocytes has been a major goal of many immuno-
logic researchers in the last few years. The identification of patients with lymphoproliferative disorders, as described in this article, provides us with a unique tool in the advancement of our understanding of the complexity of the normal immune response.

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