Immunologic Evaluation of T Chronic Lymphocyte Leukemia Cells: Correlations Among Phenotype, Functional Activities, and Morphology

By Franco Pandolfi, Giulio De Rossi, Giampietro Semenzato, Isabella Quinti, Alessandra Ranucci, Giuliana De Sanctis, Manuela Lopez, Giuseppe Gasparotto, and Fernando Aiuti

Chronic lymphocytic leukemia of T-cell origin (T-CLL), a rare variant of CLL, appears to be a clonal proliferation of mature T cells of one of several subsets. In the cells of 7 T-CLL patients, surface markers (including those reacting with a panel of monoclonal antibodies), functional activities, and electron microscopic morphology were evaluated. The phenotypic patterns of circulating T-CLL cells correspond to those of normal mature T-cell subsets. The cells of three patients demonstrated at least one marker reported to be expressed by suppressor/cytotoxic T cells; those of three patients expressed markers apparently linked with T-helper activity. Cells from one patient appeared to be a heterogeneous proliferation of more than one T-cell subset. These T-CLL cells may also retain some of the functional activity of the normal T subpopulations. Our data indicate that a combination of several tests should be used to characterize the proliferating cells in T-CLL.

Rare cases of chronic lymphocytic leukemia (CLL) result from the malignant proliferation of thymus-derived lymphocytes. Tumor cells from T-CLL patients usually show the characteristic surface markers and some functional in vitro properties of the normal mature T-cell counterpart. Thus, these proliferations provide interesting experimental models for a better understanding of the differentiation of T lymphocytes, their structure, and their functional activities.

In mice it has been possible to define functionally distinct T-cell subpopulations by different surface alloantigens that genetically identify suppressor/cytotoxic and helper T cells. Although in humans this has not been definitely concluded, it is evident that mature T-cell populations include distinct T-cell subsets programmed for restricted functions. Thus, several approaches have been attempted to identify the above subpopulations. It has been proposed that helper and suppressor T cells may be identified by several techniques, including: (A) the expression of surface receptors for the Fc portion of IgG or IgM; (B) the reactivity with monoclonal antibodies specifically directed against membrane antigens linked to a specialized function; (C) the ability to rosette with sheep erythrocytes after preincubation with theophylline; and (D) the morphological features detected by light and electron microscopy.

Since T-CLL cells seem to represent the malignant expression of fairly differentiated subsets of T lymphocytes, we undertook studies of surface markers, cytotoxic activities, and morphology of seven cases with this rare disease. The aim of this study was to achieve a better understanding of the relationships between clinical features, function, phenotype, and morphology of these putative clonal proliferations of T lymphocytes and to define possible correlations that may exist between available markers and different immunoregulatory functions of T-cell subsets.

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 percentage of lymphocytes forming conventional rosettes with sheep erythrocytes (sheep rosette-forming cells, SRFC) was determined as previously described. Stable rosettes (S-SRFC) were evaluated in the same manner, except that after 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 determined using the technique described by Pandolfi et al. Briefly, PBL were suspended in a 0.01 M solution of theophylline and incubated at 37°C for 2 hr. 50 µl of this suspension was then mixed with 50 µl of sheep erythrocyte suspension for rosetting as in the conventional assay. Cells that lacked the ability to form rosettes after preincubation, in comparison to the control test, were defined as theophylline sensitive. The formation of rosettes with mouse erythrocytes was assessed as reported. Assays to determine the frequency of cells with Fc-IgG receptors (TcR) were performed as previously described. Cells bearing receptors for human complement were analyzed using the method described by Huber and Wag zest. PBL were studied with five monoclonal antibodies: OKT6 reacts with mature thymocytes, but not with T-PBL; OKT3 reacts with all mature T cells; OKT4 and OKT8 selectively bind to subsets of T lymphocytes that exert helper and cytotoxic/suppressor activities, respectively.
helper and inducible suppressor T cells are included among 3A1-positive cells. Reactivity with the monoclonal antibodies was detected by indirect immunofluorescence.

Functional Tests

Antibody-dependent cellular cytotoxicity (ADCC) was measured according to the method described by Perlmann et al. Briefly, 0.1 ml of PBL (4 x 10^6/ml) and 0.1 ml of 3Cr-labeled chicken erythrocytes (51Cr-Ech) (2 x 10^9/ml) were added to 0.1 ml of either an anti-chicken erythrocyte IgG (final dilution 10^-4) or similarly diluted normal rabbit serum. Mixtures, in duplicate, were incubated at 37°C for 20 hr in a 5% CO₂ incubator, centrifuged, and 0.2 ml of the supernatant was analyzed for the 51Cr release by counting the emission from both supernatant and pellet in a wall-type gamma counter. The percentage of total radioactivity released from the cells into the supernatant fluid was taken as the measure of the degree of lysis. In the test for phytohemagglutinin (PHA) induced cellular cytotoxicity (PHA-ICC), PHA-P (Wellcome) was used at a concentration of 200 μg/ml.

Electron Microscopic Analysis

Cells from patients with T-CLL were isolated from the peripheral blood, washed in phosphate-buffered saline, and fixed for 1 hr at 4°C in 1% osmium tetroxide. After fixation, the cells were dehydrated, washed in phosphate-buffered saline, and fixed for 1 hr at 4°C. They were then embedded in Epon 812. Thin sections cut with an ultramicrotome were stained with uranyl acetate and lead citrate and examined with a Philips EM300 transmission electron microscope (TEM).

RESULTS

Clinical Features of T-CLL Patients

Significant clinical data of T-CLL patients are summarized in Table 1; of 7 cases, 4 were males and 3 females. The age of onset ranged from 46 to 83 yr. With the exception of case 7 who had skin lesions, the other patients were asymptomatic and diagnosis was inadvertently made by routine laboratory tests. According to the criteria proposed by Rai, diagnosis of CLL was based on bone marrow infiltration and a lymphocyte count of at least 15,000/cu mm. Two cases showed slight splenic enlargement, and in three, slight to moderate hepatomegaly was observed. Peripheral lymph nodes were palpable in three cases. Chest x-rays did not demonstrate mediastinal masses. Serum Ig levels were generally normal. In cases 6 and 7, high levels of serum IgA were detected: 362/mg dl and 570 mg/dl, respectively. Skin lesions were present in case 7 on first examination and were characterized by erythematous areas on the face, arms, and legs. Case 6 also developed nodular skin lesions in the legs after 22 mo of follow-up. Skin biopsy revealed diffuse infiltration of mature lymphocytes but no Pautrier's microabscesses. Case 1 died accidentally from a traumatic cranial injury and case 7 from bronchopneumonia. Only cases 5, 6, and 7 received treatment that consisted of prednisone and chlorambucil; these patients were studied during a period when they received no therapy.

In a separate study we have reported the characterization of a series of T-CLL cases, using a large panel of monoclonal antibodies; included in that study is the characterization of T cells from some cases in the present report. All the patients tested had no circulating OKT6 cells, while the large majority of PBL reacted with the OKT3 (Table 2), thus confirming that T-CLL cells consist of mature-appearing lymphocytes. OKT4, OKT8, and 3A1 selectively bind to different subsets of T-PBL. Cases 1 and 2 showed an extraordinary increase of OKT8^+ cells. Cases 5, 6, and 7 increased proportions of OKT4^+ cells, and 3A1^+ cells were detected in cases 2, 3, and 5. Cases 3 and 4 had significant proportions of both OKT4^+ and OKT8^+ lymphocytes.

T cells with the Fc-IgG receptor (T_G) were shown to exert suppression in in vitro systems. Data concerning T_G cells, in Table 2, demonstrated increases of these cells in cases 1 and 2.

Functional and Rosette Tests

Since it was found that T-CLL cells may express receptors for the Fc portion of IgG, the cytotoxicity against antibody-coated targets and PHA-induced cellular cytotoxicity were tested. The cells of cases 1 and 2, which have the OKT3^+, OKT8^+, T_G phenotype, and those of cases 3 and 7, mediated ADCC (Table 2). In contrast, the cells of case 6 (OKT4^+) had reduced

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex</th>
<th>Age</th>
<th>Time (mo)</th>
<th>Rai Stage</th>
<th>Medial Mass</th>
<th>Skin Lesions</th>
<th>WBC (x 10⁹/cu mm)</th>
<th>Percent Lymphocytes PB</th>
<th>Percent Lymphocytes BM</th>
<th>Survival Time* (mo)</th>
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<tbody>
<tr>
<td>1. P.T.</td>
<td>M</td>
<td>83</td>
<td>+ + +</td>
<td>3</td>
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<td>15.0</td>
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<td>33.0</td>
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<td>4. E.D.F.</td>
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<td>62.0</td>
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<td>5. A.V.</td>
<td>M</td>
<td>67</td>
<td>+ +</td>
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<td>45.2</td>
<td>86</td>
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<td>6. E.O.</td>
<td>M</td>
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<td>7. N.C.</td>
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PM, peripheral blood; BM, bone marrow; (-) absent; (+) slight; (+ +) moderate; (+ + +) massive.

*Calculated from date of diagnosis.

Table 1. Significant Clinical and Laboratory Data in Seven T-CLL Cases at Diagnosis and Survival Time
ADCC activity, as did those of case 4, which were of mixed phenotype. On the other hand, PHA-induced cellular cytotoxicity was strongly present in the cells of cases 2 and 7, reduced in those of cases 1, 2, and 6, and undetectable in those of patient 4.

T-CLL cells from seven patients were also studied using five different rosette techniques. The cells of all patients formed rosettes with sheep erythrocytes (sheep rosette-forming cells or SRFC) as expected in T-CLL. On the other hand, the cells did not form rosettes with mouse red cells (a property of a subpopulation of B cells) and did not have C3b receptors. The formation of stable rosettes with sheep erythrocytes (S-SRFC) is a property of immature T cells (thymocytes and T blasts from patients with T acute lymphoblastic leukemia), but mature lymphocytes lack this marker. S-SRFC are also formed by a subpopulation of lectin-activated mature T lymphocytes. In our series of patients, S-SRFC were detected in PBL from three cases, two of them with normal to high proportions of OKT4 cells. On the other hand, theophylline-sensitive cells were increased in case 4 and in case 2, the latter being an almost pure proliferation of OKT8, Tg cells.

**Ultrastructural Features of T-CLL Cells**

Representative TEM features of cells observed in T-CLL cases are reported in Figs. 1–7. Figure numbers correspond to case numbers. Significant data are summarized in Table 4. The nucleus was round in cells from cases 1, 2, 3, and 4 and indented or convoluted in the OKT4⁺ cells (cases 5, 6, and 7). Nucleoli were preeminent in the OKT4⁺, 3A1 lymphocytes. Cytoplasmic membrane-limited electron-dense granules were observed in the large majority of cells isolated from two cases (1 and 4), whereas in cytoplasm of some lymphocytes of case 3, we detected some membrane-bound organelles containing smaller vesicles (Fig. 3, insert A). The Golgi apparatus was well developed in cells from cases 3, 4, and 5.

**Discussion**

In the present study we characterized seven cases of T-CLL by reactivity with monoclonal antibodies, surface markers, functional activities, and morphology at the TEM level. It has been reported that T-CLL cells are usually the result of the proliferation of a subset of mature-appearing T cells. The patient’s cells were also used as reagents to define the specificity of techniques proposed for the identification of T-cell subpopulations with immunoregulatory activity, and to evaluate the correlations between the results obtained with different markers. Our data clearly indicate that T-CLL is a heterogeneous disease; the cells of the different cases express surface markers that correspond to those of normal mature T-cell subsets. According to the reactions with the monoclonal antibodies, the cells of two patients (cases 3 and 4)
Fig. 1. TEM micrograph (x 18,900) of patient 1 cells showing round nucleus, abundant cytoplasm with mitochondria, and few membrane-bound electron-dense granules.

Fig. 2. TEM micrograph (x 16,500) of patient 2 cells showing high nucleo-cytoplasmic ratio and several mitochondria.

Fig. 3. TEM micrograph (x 17,000) of patient 3 cells. Cytoplasm contains mitochondria and Golgi cisternae. Some cells had membrane-bound organelles containing smaller vesicles (insert A. x 35,000). Autophagosomes (insert B. x 31,000) were a remarkable feature of some of these T-CLL cells.

Fig. 4. TEM micrograph (x 16,000) of patient 4 cells, showing abundant cytoplasm, numerous mitochondria, profiles of rough endoplasmic reticulum, a well developed Golgi apparatus, and few scattered electron-dense granules.

appeared to be the result of the proliferation of more than one clone, since both T4⁺ and T8⁺ cells were detected. The cells in five cases consisted of pure proliferations of a given subset, since virtually all the cells were either T4⁺ or T8⁺. In Fig. 8 we summarize the relationship between SRFC and surface antigens identified by monoclonal antibodies on normal PBL.⁸,²³ The cells from these latter five patients are accordingly grouped in Fig. 8, thus showing that T-CLL may originate from each individual subgroup present in normal donors.

Among the five patients who appear to have a pure proliferation of a given subset, cells from two patients expressed the T8 antigen, related to the cytotoxic/suppressor T-cell function, and those from three cases had the T4 helper-related antigen. These T4⁺ cells may be further subdivided according to their reactivity with the 3A1 reagent. In the two T4⁺, 3A1⁺ patients (cases 6 and 7), skin lesions were observed. This is noteworthy since it has been reported that Sézary's syndrome and mycosis fungoid circulating T cells express the T4⁺, 3A1⁺ helper T-cell phenotype.²³ Although skin biopsy was negative for Pautrier's microabscesses in our cases, TEM features of these cells revealed some characteristics of the Sézary cells, in particular the convolutions of the nucleus and the presence of a prominent nucleolus. However, these cells do not appear to be typical Sézary cells because: (1) margination of heterochromatin was not evident as in typical Sézary cells and (2) glycogen accumulation²⁴ and fibrillar structures²⁵ were not present. These find-
Findings are consistent with the possibility that T4⁺, 3A1 helper T cells may proliferate or preferentially “home” in the skin. It needs to be clarified whether the T4⁺, 3A1 T-CLL and Sézary’s syndrome represent two distinct diseases. A remarkable clinical observation, suggesting a possible in vivo helper activity of these T-CLL cells, was the presence of high levels of serum IgA in both these two patients.

Lymphocytes from case 5 showed the OKT4⁺, 3A1⁺ phenotype (Fig. 8), and TEM features of these cells are clearly different from those of the typical Sézary syndrome.
cell. At diagnosis, he showed CLL classified as stage 3 according to Rai, and therefore received treatment. In all, we studied ten cases of T-CLL. Seven cases are reported here; two have been described previously, and one case has not been published. Of the ten cases observed, three were OKT4+ and these same three cases were the only ones requiring therapy: in two of them (cases 6 and 7) to control the skin involvement.

Previous reports have shown that, in humans, T-suppressor cells are included among: (A) Fc-IgG receptor positive cells (T8); (B) cells reactive with the monoclonal antibody OKT8; (C) theophylline-sensitive rosetting cells; and (D) cells with a certain TEM morphology (large cells with an abundant cytoplasm, a well developed Golgi apparatus, and unit membrane-bound granules in their cytoplasm). Moreover, at least T8 cells are thought to mediate ADCC activity. Such markers were not detected in the three OKT4+ T-CLL cells (cases 5, 6, and 7). Some significant properties of lymphocytes in cases 1, 2, 3, and 4 are summarized in Table 5, where the suppressor activity of these cells on lectin-induced responses of normal PBL and on B-cell differentiation is also reported.

Cells from patients 1 and 2 exhibited in vitro suppressor activity. In case 1, the cells had the T8 antigen, expressed Fc-IgG receptors, and had cytoplasmic granules at the TEM level. However, theophylline-sensitive cells were undetectable. The cells from patient 2 had the T8 antigen, Fc-IgG receptors, and this patient had high levels of theophylline-sensitive cells. Yet, lymphocytes from this case lacked cytoplasmic granules. As in a previously reported T-CLL case, cells from patient 2 had undetectable natural killer (NK) activity (data not shown) despite a strong ADCC.

On the other hand, cells from patients 3 and 4 had predominately OKT4+ cells in their peripheral blood, but also had significant proportions of OKT8+ cells. Although the cells of these two patients appeared to be the result of the proliferation of more than one clone, according to the reactivity with monoclonal antibodies, TEM showed the presence of cytoplasmic granules in almost all the lymphocytes of case 4. Thus, these data suggest that the proliferating cells in case 4 may be a morphologically homogeneous clone. In case 3, granules were not evident, but in the cytoplasm of some of these cells, membrane-bound organelles containing small vesicles (Fig. 3, insert A), were detected. These cells did not have suppressor activity but were ADCC positive.

The overall analysis of our data suggests that the presence of high proportions of T8 and OKT8+ cells appears to correlate with the detection of suppression in in vitro tests (Table 5). Theophylline-sensitive cells were a property of lymphocytes isolated from patient 2 (who was found to have suppressor activity), but were also isolated from patient 4, who lacked suppressor and cytotoxic functions. Patient 1, lacked theophylline-sensitive cells, while these cells demonstrated suppressor functions. Results of morphology were also intriguing. Cells from patient 1 had the typical morphology of T8 cells; showed Fc-IgG receptors, were OKT8+, and had suppressor and cytotoxic activities. However, in the other cases we observed a lack of correlation between the presence of granules and functional activities. Cells from case 2 suppressed, while lacking cytoplasmic granules. The reverse was seen in patient 4. His cells lacked suppressor activities, while showing...
cytoplasmic granules. Recently, the term large granular lymphocytes (LGL) has been introduced to describe the morphology of a mononuclear cell characterized by a large cytoplasm, a well developed Golgi apparatus, and the presence of distinctive cytoplasmic granules. It has been reported that these LGL may mediate NK function. Therefore, it remains to be seen if the two cases of LGL T-CLL reported by us (patients 1 and 4) mediate NK functions.

At the present time no markers for clonality are readily available for T cells. Therefore, the diagnosis of malignancy cannot be unequivocally confirmed in our cases. The study of the reactivity with monoclonal antibodies may substantiate the clonal proliferation of a given subset in the majority of T-CLL cases. Brouet reported T-CLL cases characterized by a mixed proliferation according to their reactivity with monoclonal antibodies, and these findings are consistent with two of our cases (3 and 4). These cases may represent initial phases of the disease. With such limitations, T-CLL cells represent good reagents for evaluating the specificity of techniques proposed for the identification of regulatory T-cell subsets with a given in vitro activity. Our data indicate that only a combination of several surface markers, functional tests, and morphology may help in the characterization of these cells.

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