Natural Killer and Suppressor T-Cell Chronic Lymphocytic Leukemia

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A patient with low-grade lymphocytosis, splenomegaly, and neutropenia, but adequate myeloid leukogenesis, was found to have chronic lymphocytic leukemia, which represented a clonal proliferation of a distinct T-lymphocyte subset. The lymphocytes did not form E rosettes but had an OKT3+, OKT4+, OKT6+, OKT8+, OKT11+, HNK-1+, HNK-36+, OKLa1+, OKM1+ phenotype and functionally had suppressor and natural killer activity. Morphologically, they were large granular lymphocytes, which were strongly acid phosphatase positive and nonspecific esterase negative. They did not respond to mitogens or to alloimmune cells. Initially, the spleen appeared to be the most involved organ and, judging from the high proportion of leukemic splenic lymphocytes in the S and G2/M phases of the cell cycle, was also the organ of origin of the leukemic cells. Only a few leukemic cells in the blood and bone marrow were in S and G2/M phases. After splenectomy, the lymphocyte count rose considerably and the bone marrow became progressively more infiltrated by tumor nodules. One year after diagnosis, the patient was started on chemotherapy because of progressive anemia. He responded to the chemotherapy by normalization of the hemoglobin and neutrophil count and had a moderate decrease in the bone marrow involvement and peripheral lymphocytosis.

T-cell chronic lymphocytic leukemia (T-CLL), a relatively rare disorder in the United States and Europe but more common in Japan, appears to be a heterogeneous disease by clinical, morphological, and immunologic criteria.1-17 This heterogeneity suggests that the various distinct T-CLLs represent clonal proliferation of cells arising from several different T-cell subsets. Thus, the functional activity and cell surface antigen phenotype of T-CLL cells from a single subject may represent those of a distinct subpopulation of normal T cells.

Because of the frequently benign clinical course, lack of traditionally accepted histologic evidence of malignancy, and controversy about what constitutes a monoclonal T-cell population, there has been disagreement about the definition of T-CLL.16,17 The term T γ lymphocytosis is preferred by some.15,17

In this article we describe the clinical, histologic, and cytologic characteristics of a case of T-CLL in which the leukemic cells exhibited natural killer (NK) activity, suppressed mitogen-stimulated lymphocyte proliferation, and B-lymphocyte immunoglobulin synthesis. They expressed monoclonal antibody-defined cell surface differentiation antigens characteristic of mature peripheral blood T cells (OKT3), suppressor/ cytotoxic T cells (OKT8), and natural killer cells (HK-1). The characteristics of these T-CLL cells point to the probable existence of a subset of normal T cells with both NK and nonspecific suppressor cell activity.

MATERIALS AND METHODS

Morphology, Cytochemistry, and Histochemistry

Blood and bone marrow smears and splenic imprints were stained with Leishman's stain and periodic acid-Schiff (PAS) reaction and were examined for acid phosphatase, alpha-naphthyl acetate esterase (nonspecific esterase), and alkaline phosphatase activity. Sections of bone marrow biopsies, spleen, liver, and lymph node were stained with hematoxylin and eosin. The spleen was also stained for iron and naphthol chloroacetate esterase (NCA) for the detection of neutrophils. Peripheral blood cells were also examined by electron microscopy.

Immunologic Studies

Mononuclear cells, separated by Ficoll-Hypaque technique from peripheral blood, bone marrow, and spleen, were tested for surface immunoglobulins, rosette formation with sheep erythrocytes (E rosettes), receptors for complement and the Fc portion of IgG, mitogen- and alloantigen-induced proliferation, and HLA-A,B,C, and DR phenotype using methods previously described.18-20 For mitogen-stimulated cultures, a cell concentration of 1.5 × 10⁶/ml and 10 μg of phytohemagglutinin (PHA), pokeweed (PW), or concanavalin-A (Con-A) were used. Each culture well contained 3 × 10⁵ cells. For the alloantigen-stimulated cultures, the cell concentrations of both responder and stimulator cells were 10⁶/ml. Each well contained 5 × 10⁴ responder cells (irradiated with 2,500 rads) and 5 × 10⁵ responder cells. To determine whether a small percentage of normal lymphocytes in the patient's blood could be responsible for the response to mitogens, mononuclear cells from a normal control and previously frozen cells of the patient from 4/19/82 were diluted to 5%, 10%, 20%, 30%, 40%, 50%, and 75% of the usual cell concentration and the usual amounts of mitogens were added. For the same purpose, mixed lymphocyte cultures were performed with either stimulator or responder cells decreasing by 10,000 cells per well. E-rosette formation was also tested after neuraminidase treatment of the sheep red cells and leukemic cells.21 Indirect immunofluorescence was used to enumerate the proportions of lymphocytes in blood, bone marrow, and spleen suspensions that expressed antigens detected by the following monoclonal antibodies: OKT3, OKT4, OKT6, OKT8, OKT10, OKT11, OKM1, and OKLa1 (Ortho Diagnostics, Raritan, NJ), HNK-1 (Becton Dickinson, Sunnyvale, CA), and HNK-36 (Kaplan: manuscript in preparation). Leukemic cells were examined for suppressor activity by determining their...
effect on normal lymphocyte mitogen and alloantigen-induced blasto-
genesis and pokeweed mitogen and antibody-dependent cell-mediated cytoxicity (ADCC) against antibody-coated B-lymphoblast cell line SB were tested as previously described. The phenotype of cells mediating NK activity was determined by treating aliquots of cells with saturating amounts of OKT3 or HNK-36 antisera or diluent for 30 min at 37°C, followed by 2 sequential treatments with rabbit complement. The cells were then resuspended to the original volume and tested for NK activity.

Phagocytic and Cytogetic Studies

Phagocytic studies using latex particles were performed according to the method of Daniel et al., and preparations were examined with the electron microscope. Cytogetic studies were done on PHA-, Con-A-, and PW-stimulated cultures of peripheral blood lymphocytes after 72 hr of incubation.

DNA Content Analysis

DNA histograms of the leukemic cells in blood, bone marrow, and spleen stained with propidium iodide were prepared on a Coulter TPS-1 cell sorter and analyzed according to the program by Dean and Jett. Chick erythrocytes were used as an internal standard. Controls consisted of normal blood mononuclear cells (12), reactive lymph nodes (11), tonsils (7), spleens (2), and bone marrows (3).

CASE HISTORY

A 48-yr-old man was admitted for evaluation of jaundice and possible hemolytic anemia. Ten months earlier he had undergone a cholecystectomy for cholelithiasis, but no other abnormalities had been noted at that time. Except for slight scleral icterus, there were no abnormalities on the current physical examination. Significant laboratory findings were hemoglobin of 10.3 g/dl, reticulocyte count of 3.3%, neutropenia ranging from 500 to 1,500/cu mm, and absolute lymphocytosis of up to 6,000/cu mm. Platelets were within the normal range. Lactic dehydrogenase was 242 IU (normal, 100-225 IU), bilirubin was 3.2 mg/dl (normal, 0.2-1.2 mg/dl) with 1.6 mg/dl indirect. The Coombs test, direct and indirect, was negative. Liver-spleen scan demonstrated enlargement of the spleen, and an abdominal tomogram revealed a mass in the region of the head of the pancreas. During exploratory laparotomy, splenectomy and liver biopsy were performed, and slightly enlarged lymph nodes near the pancreatic head were removed. The spleen weighed 480 g. Postoperatively, icterus improved and hemoglobin and granulocyte count began to increase. Although hemoglobin remained stable near 14 g/dl, the neutrophil count again dropped to 1,100/cu mm and the indirect bilirubin remained mildly elevated. The absolute lymphocyte count rose immediately and then progressively increased to 24,000/cu mm over a period of 5 mo. Immunglobulins were within the normal range. Eleven months after diagnosis, the patient's hemoglobin started to drop and the bone marrow was found to be more involved. Chemotherapy with cytoxan, onco-

RESULTS

Morphology, Cytochemistry, and Histochemistry

Blood. Most of the lymphocytes were large and uniform in size. The cytoplasm was clear and abundant and contained many azurophilic granules. The nucleus in the majority of cells was regular, had dense chromatin, and no visible nucleolus (Fig. 1). Membrane-bound lysosomes and occasional phagolysosomes were evident on electron microscopy (Fig. 2). The acid phosphatase reaction showed numerous scattered granules. Nonspecific esterase activity was absent in the leukemic cells. PAS reaction was positive in small granules, especially near the cell membrane. The leukocyte alkaline phosphatase reaction was negative. Following chemotherapy, occasional immature lymphocytes were seen in the peripheral blood.

Bone marrow. The initial bone marrow was hypercellular; myeloid leukogenesis and megakaryocytophesis was increased. There were numerous mature neutrophils and their precursors. Lymphocytes accounted for 29% of the cells and morphologically resembled those in the blood. In sections there was only a slight diffuse increase in lymphocytes. Another bone marrow sample taken 4 mo later contained 51% lymphocytes and some lymphoid nodules. A third bone biopsy 9 mo later showed more extensive involvement, including paratrabecular localization of lymphoid nodules. Eleven months later, there were many large lymphoid nodules. A fifth bone marrow examination after chemotherapy had 44% lymphocytes, an adequate number of granulocytes, and no distinct lymphoid nodules.

Spleen. Sinusoids were filled with small regular lymphocytes (Fig. 3). In addition, there were large collections of lymphocytes around small vessels as well as around larger trabecular vessels (Fig. 4). The naphthol chloroacetate esterase reaction demonstrated numerous neutrophils, bands, and occasional myelocytes in sinusoids and in a few lymphoid aggregates. There was a great increase of hemosiderin throughout the spleen. In splenic imprints, the cells were morphologically and cytochemically identical to those in the blood.

Fig. 1. Leukemic lymphocytes with abundant cytoplasm and cytoplasmic granules (x2,016).
NK AND SUPPRESSOR T-CLL

Fig. 2. Leukemic lymphocytes after incubation with latex particles, showing membrane-bound lysosomes and phagolysosomes but no ingested latex particles (×5,629).

Lymph node. The architecture was preserved. The paracortical area appeared moderately enlarged, but a diagnosis of malignancy could not be made because of the relatively normal appearance of the cells.

Immunologic Studies

Results of the immunologic tests are listed in Tables 1–3. Few lymphocytes formed E-rosettes with untreated sheep red cells. There was no significant increase in E-rosette formation following neuraminidase treatment of either the sheep red cells or mononuclear cells. Most of the cells, however, reacted with anti-OKT11, a monoclonal antibody presumably specific for sheep erythrocyte receptors. The fluorescence, however, was much weaker than that on normal cells. Following chemotherapy, there was a significantly higher percentage of E-rosetting cells. In addition, most of the cells reacted with anti-OKT3, specific for mature peripheral T cells, anti-OKT8, an antibody to suppressor/cytotoxic T cells, and anti-HNK-1 and anti-HNK-36, both specific for NK cells. Few or no cells reacted with antisera OKT4, specific for helper/inducer T cells, OKT10, reactive with activated lymphocytes and other precursor cells, OKM1, reactive with some monocytes and some natural killer and killer cells, and OKIa, also reactive with monocytes, B lymphocytes, and some activated T cells. Most of the leukemic cells had receptors for the Fc portion of IgG as determined by rosette formation with IgG-coated ox erythrocytes (IgGEA). There were only a few cells with surface immunoglobulins and receptors for complement (IgMEAC). HLA typing revealed the expression of HLA-A,B (A2; B8, 18) antigens, but not DR antigen.

Table 2A indicates as few as 5% of normal lymphocytes produced the same degree of response to mitogens as the patient's cells. On the contrary, dilution of the patient's cells resulted in a marked reduction of the responses to mitogens. Reduction of normal stimulator or responder cells to 10⁴/well also resulted in ³H-thymidine uptakes similar to those seen in the patient's blood: 10⁴ stimulator cells and 5 × 10⁴ responder = 5,492 cpm; 10⁴ responder and 5 × 10⁴ stimulator cells = 2,638 cpm. Thus, it is likely that the patient's lymphocyte response to mitogens and allogeneic cells (Table 2B) was due to the presence of even a very small percentage of normal lymphocytes. The patient's cells, however, did suppress mitogen-induced (Table 2C), but not alloantigen-induced, blastogenesis of normal lymphocytes (not shown). Secretion of immunoglobulin by pokeweed-stimulated normal B lymphocytes was also suppressed by the leukemic cells (Table 2D).

As shown in Table 3, the patient's cells had moderate NK and K cell activity, as measured, respectively, by spontaneous lysis of K562 target cells and antibody-
Table 1. Immunologic Studies

<table>
<thead>
<tr>
<th>Date</th>
<th>Tissue</th>
<th>Percent Lymphocytes in Mononuclear Preparation</th>
<th>Rosettes (%)</th>
<th>Reactions With Monoclonal Antisera (%)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>E IgG A</td>
<td>IgM E A OKT 3 OKT 4 OKT 6 OKT 8 OKT 10 OKT 11 OKT a 1 OKT M 1 HNK - 1 HNK - 36</td>
</tr>
<tr>
<td>12/4/81</td>
<td>Spleen</td>
<td>95</td>
<td>18 65 15</td>
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<td>83 13 0 73</td>
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<td>69 15 0 56</td>
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<td>48 ND ND</td>
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<td>30 28 50 36  22 0.5 16 5 25 48 13</td>
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*Seven weeks after chemotherapy.

Table 2. Functional Studies

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<tr>
<th>Date</th>
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<th>Percent Lymphocytes in Mononuclear Preparation</th>
<th>Rosettes (%)</th>
<th>Reactions With Monoclonal Antisera (%)</th>
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<td></td>
<td></td>
<td></td>
<td>E IgG A</td>
<td>IgM E A OKT 3 OKT 4 OKT 6 OKT 8 OKT 10 OKT 11 OKT a 1 OKT M 1 HNK - 1 HNK - 36</td>
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<tr>
<td>A. 4/19/82</td>
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<td></td>
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<tr>
<td>B. 3/3/82</td>
<td>Mixed</td>
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Table 3. NK and K Activity of Patient's Cells

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<th>Treatment</th>
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<tr>
<td></td>
<td>K</td>
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<tr>
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<tr>
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<td>182</td>
<td>16</td>
</tr>
<tr>
<td>OKT 3 + complement</td>
<td>214</td>
<td>5</td>
</tr>
<tr>
<td>HNK-36 + complement</td>
<td>104</td>
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</table>

*Number of cells/10^5 cells required to give specific lysis of 5 x 10^5 target cells as calculated from the exponential fit equation.26

Phagocytosis and Cytogenetics

No ingestion of latex particles or red cells was noted. No chromosomal abnormalities were present in 43 cells analyzed by Giemsa and Giemsa-banding techniques.

DNA Content Analysis

In the blood sample from 12/11/78 (Fig. 5A), 99% of the cells were in G_0/G_1 phase. In the bone marrow from 12/15/81, a small percentage of cells were in S phase (1.27%) and 0.8% in the G_2/M phase (Fig. 5B).

coated B-lymphoblast cell line SB. The NK activity appeared to be due to leukemic cells rather than residual normal NK cells present in the peripheral blood mononuclear cell suspension. Unlike normal NK cells, which, as expected, were not affected by complement lysis with anti-OKT3, the cells from our patient showed decreased NK activity after such treatment. By contrast, both the leukemic and normal cells showed decreased NK cell activity after treatment with the NK-specific monoclonal antibody, HNK-36.

Phagocytosis and Cytogenetics

No ingestion of latex particles or red cells was noted. No chromosomal abnormalities were present in 43 cells analyzed by Giemsa and Giemsa-banding techniques.
The mononuclear preparation of the bone marrow contained a small percentage of myeloid and erythroid precursors. In contrast, although 95% of the splenic cells were lymphoid and contained no granulocyte precursors, a considerable number of cells were in S (2.92%) and G2/M (3.74%) phases (Fig. 5C). There was no evidence of aneuploidy.

Control values for G0/G1 phase for normal blood, bone marrow, spleens with reactive follicles, reactive lymph nodes, and tonsils were 98.43% ± 0.74%, 88.55% ± 1.53%, 94.96% ± 1.21%, 95.63% ± 1.50%, and 92.45% ± 1.87%, respectively; for the S phase: 0.82% ± 0.37%, 6.70% ± 0.82%, 2.93% ± 1.80%, 2.98% ± 1.26%, and 3.66% ± 0.64%, respectively. The G2/M component for lymph nodes and tonsils was not considered because of the difficulty of getting a good...
single-cell suspension. In blood, bone marrow, and spleen, the G1/M phases were 0.75% ± 0.55%, 4.75% ± 0.94%, and 2.12% ± 0.60%, respectively.

Thus, the phases of the cell cycle of our patient's blood cells resembled normal inactive peripheral blood cells. In the patient's bone marrow, where 73% of cells were lymphoid, the S and G1/M phases were lower than in control marrows. In the control spleens, where there was moderate follicular hyperplasia, the S and G1/M phases were comparable to those of our patient. Since our patient's spleen did not have reactive follicles but contained primarily T lymphocytes, it is likely that the S and G1/M components were from the T-cell population.

**DISCUSSION**

T-cell chronic lymphocytic leukemia is morphologically, clinically, and immunologically a heterogeneous disease, reflecting the diversity of normal peripheral T cells. The leukemic cells described here appeared to represent the clonal proliferation, probably originating in the spleen, of a distinct T-lymphocyte subset with natural killer and suppressor phenotype and activity. Evidence for clonality rests on the observation that the cells were uniformly E-rosette-negative, had Fc receptors, and expressed the distinctive antigenic phenotype OKT3⁺, OKT4⁺, OKT6⁺, OKT8⁺, OKT10⁺, OKT11⁺, HNK-1⁺, HNK-36⁺, OKIa1⁻, OKM1⁻, and lacked nonspecific esterase. T-cell lineage was evident from the expression of T-cell-specific antigens OKT3, OKT8, and OKT11. Despite reactivity with anti-OKT11, a monoclonal antibody thought to be specific for sheep erythrocyte receptors, the cells failed to bind sheep erythrocytes. Since the fluorescent intensity of the OKT11 antigen was low, this may imply that the antigen is poorly expressed on the leukemic cells or that it is an epitope, which is also present on molecules that do not function as receptors for sheep erythrocytes. West et al. have described a poor binding efficiency for sheep red cells by natural killer cells. The leukemic T cells had several characteristics associated with normal NK and K cells. Morphologically, there were large, granular, acid-phosphatase-positive lymphocytes. However, they lacked nonspecific esterase, an enzyme previously associated with large granular lymphocytes. The cells expressed the NK-specific antigen, HNK-1, and the analogous antigen, HNK-36, detected by monoclonal antibodies that one of us (J.K.) has recently developed. They moderately lysed standard NK target cell line K562. Although the NK activity against this target was relatively low, it was almost certainly due to the OKT3⁺ leukemic T cells rather than residual normal NK cells, since it was abrogated by complement lysis of OKT3⁺ cells, a treatment that fails to reduce NK activity of normal peripheral blood lymphocytes. The cells also showed modest K cell ADCC activity, as measured by lysis of the antibody-coated B-lymphoblast cell line SB. Given that more than 90% of this patient's circulating mononuclear cells were leukemic cells with phenotypic properties of NK cells, it is of interest that the NK and K activities of these cells were not higher than that of normal peripheral blood lymphocyte suspensions, which usually contain less than 15% NK and K cells. The relatively low NK activity could be explained if NK cells are clonal in their specificity, and the leukemic cells represent a clone of normal NK cells specific for an antigen present in only low density on cell line K562. However, this would not account for their relatively low K activity. Alternatively, the OKT3⁺ subpopulation of NK and K cells, which Platsoukas has recently documented and from which these leukemic cells apparently originate, may be one with relatively low lytic capacity. The Tγ lymphocytes from five patients in Rümke et al. series had either only suppressor or K cell, or NK and K cell activity. Abo et al. recently demonstrated that HNK'T⁺ cells had low NK activity and exhibited virtually no response to either mitogens or allogeneic cells. The responses to mitogens and allogeneic cells by our patient's cells could be explained by the presence of a very small number of normal cells, as shown by titration experiments.

In addition to having some NK and K cell lytic activity, our patient's leukemic cells were also able to suppress blastogenesis and immunoglobulin secretion by mitogen-stimulated normal B lymphocytes. This suppressor activity corresponds to the expression of OKT8, an antigen associated with suppressor and cytotoxic T cells. The findings imply that at least some normal NK cells, particularly those that express OKT8, may also function as nonspecific suppressor cells.

Other cases of T-CLL with the suppressor/cytotoxic OKT3⁺, OKT8⁺ phenotype and Fcγ receptors have been previously reported. They have varied in morphologic, functional, and clinical characteristics. Rümke et al. present a good review of these cases. Schlimok et al. reported a case of CLL that was characterized by the OKT3⁺, OKT8⁺ phenotype, but also had the OKM1 antigen. The leukemic cells, large granular lymphocytes, had NK and K activity to Molt-4 and human melanoma cell lines, but unlike the cells of our patient, failed to suppress B-cell differentiation in vitro. The cells also differed from ours in that they were both acid phosphatase and nonspecific esterase positive. They may thus represent a different type
of NK cells, in this case, without suppressor activity.

The spread of tumor was of interest in its limited nature. The initially examined bone marrow had a slight increase in the number of lymphocytes, and leukemic involvement could be proven only immunologically. The biopsied lymph nodes were studied only histologically and a diagnosis of malignancy could not be made. The liver showed no involvement of portal areas. Leukemic cells were present in sinusoids. The spleen was moderately enlarged (480 g) and seemed to be the most prominently affected organ. The neoplastic cells were found in sinusoids and a moderately expanded white pulp. It was impossible to determine whether T or B zone areas were involved. Without immunologic studies, which showed that almost 90% of lymphocytes were T cells, it would have been difficult to determine that the majority of cells of the white pulp were malignant. The large number of neutrophils and the excessive iron deposits attested to the fact that the spleen was, at least in part, responsible for the trapping of red cells and granulocytes.

Although the indirect bilirubin remained slightly elevated, the patient’s hemoglobin initially returned to normal following splenectomy, but the neutrophil count remained just below the lower limit of normal. Eleven months later, the hemoglobin again dropped to 10 g/dl. An antibody to red cells could not be demonstrated by Coombs’ test. The cause of the neutopenia, a phenomenon also seen in most suppressor T-CLL, is unresolved. Preliminary experiments revealed that the patient’s leukemic cells failed to inhibit the proliferation and differentiation of myeloid stem cells, as measured by in vitro myeloid colony formation. This fits with the fact that the bone marrow contained abundant erythrocyte and granulocyte precursors as well as mature granulocytes. Moreover, the increase of granulocytes and iron in the spleen is evidence of the ability of the mature cells to leave the bone marrow. Cellular immunity and production of antibodies to mature granulocytes are potential mechanisms of neutropenia that have not, as yet, been tested. Chemotherapy, instituted when the hemoglobin again dropped to 10 g/dl and the bone marrow showed numerous lymphoid nodules, resulted in improvement of the hemoglobin and bilirubin level, a return of the neutrophil count to normal, and a slight drop in the T lymphocytosis with a change in the surface markers, especially a large percentage of E-rosetting cells. This may reflect the effects of chemotherapy or an emergence of normal T cells.

In some chronic T-lymphocyte proliferations, especially those with only slightly elevated peripheral lymphocyte counts, the malignant nature of the proliferation has been questioned because monoclonality of the T-cell population or histologic evidence of malignancy could not be unequivocally demonstrated. In the present case, the cells morphologically, cytochemically, and phenotypically were distinctive enough to suggest the diagnosis of malignancy despite the initial low-grade lymphocytosis and scant tissue involvement. This was confirmed 9 and 11 mo later by a progressive increase in lymphoid tissue and by the paratrabecular lymphoid infiltration in the bone marrow, characteristic of malignant lymphoid neoplasms, as well as a subsequent drop in the hemoglobin.

The significantly greater leukemic lymphocytosis shortly after splenectomy is of interest. The spleen may have been the preferential homing site as well as the organ of origin. This is suggested by the presence of a significant percentage of splenic, but not bone marrow or blood, lymphocytes in S and G2/M phases, suggesting cellular synthesis in the spleen but not in blood and bone marrow.

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Natural killer and suppressor T-cell chronic lymphocytic leukemia

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