T Cell Chronic Lymphocytic Leukemia:
Presence in Bone Marrow and Peripheral Blood of Cells That Suppress Erythropoiesis In Vitro

By Ronald Hoffman, Samuel Kopel, Shu Dean Hsu, Nicholas Dainiak, and Esmail D. Zanjani

The pathogenesis of the anemia associated with malignancy was investigated in a patient with T cell chronic lymphocytic leukemia. The plasma clot culture system was used as a measure in vitro of erythropoiesis. The patient’s peripheral blood and marrow T lymphocytes obtained both before and after transfusion therapy suppressed erythroid colony formation by normal human bone marrow cells. Pretreatment of the patient’s bone marrow T cells by antithymocyte globulin (ATG) and complement reversed this suppression. In addition, pretreatment of the patient’s marrow cells with ATG and complement markedly augmented erythropoiesis in vitro. The expression of erythroid activity caused by the selective destruction of the suppressor T lymphocytes in the patient’s bone marrow with ATG and the suppression of normal erythropoiesis by the patient’s bone marrow and peripheral blood lymphocytes suggest that interaction between the malignant T cell and the erythropoietin-responsive stem cell is important in the production of anemia in this patient.

Anemia occurs in over 50% of patients with chronic lymphocytic leukemia (CLL). This anemia can on some occasions be attributed to a shortened red cell survival due either to the effects of an autoantibody and/or to an enlarged spleen. However, in the great majority of anemic patients with CLL reduced red blood cell production is the major factor in the production of anemia.

To date, most cases of CLL have been shown to result from the proliferation of bone marrow–derived or bursa-equivalent B lymphocytes. In only a small number of cases of CLL has the proliferating cell population been defined as a thymus-derived T lymphocyte.

We studied in depth a patient with T cell CLL whose course was characterized by severe anemia due to the underproduction of red blood cells. Using cell culture techniques that allowed the proliferation and differentiation in vitro of erythropoietin-responsive stem cells, we were able to show that the anemia of this individual may have been the consequence of an interaction between hematopoietic stem cells and malignant T lymphocytes.

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CASE REPORT

The patient, a 74-yr-old white male, complained in January 1977 of dizziness, weakness, and shortness of breath. Physical examination showed marked pallor but no detectable lymphadenopathy. Spleen was palpated 2 cm below the left costal margin, while the liver was detected 4 cm below the right costal margin. There was no evidence of skin nodules, plaques, or erythroderma. Laboratory testing revealed hematocrit 21%, Hb 7.5 g/dl, white blood cell count 18,000/μl, reticulocyte count 0.1%. The white blood cell differential was 75% lymphocytes, 15% segmented neutrophils, 8% monocytes, and 2% eosinophils. The red blood cells appeared normochromic and normocytic. The lymphocytes in the peripheral blood were small, uniform, and mature appearing with a high nuclear/cytoplasmic ratio. The chromatin was heavily clumped, and indistinct nucleoli were noted. The lymphocytes were easily ruptured while making smears, and many "smudge" cells were seen in these smears. The bone marrow aspirate was hypercellular with severe erythropoietic hypoplasia. Normal numbers of megakaryocytes were noted. The marrow was diffusely infiltrated with lymphocytes. About 55%, 60% of bone marrow cells were lymphocytes. Bone marrow iron stores were increased. Cytogenetic studies of a direct bone marrow specimen showed a normal male karyotype. The direct Coombs' test was negative. The serum haptoglobin, vitamin B12, and folate levels were within normal range. Studies of liver and renal functions were unremarkable. There was no evidence of blood loss into the gastrointestinal tract. Quantitation of the serum immunoglobulins showed IgG 1050, IgA 115, and IgM 90 mg/dl. No monoclonal protein abnormality was detected. An antinuclear antibody test was negative. The patient required transfusions of 1-2 units of packed red cells every 2 wk. Chemotherapy with cytoxan, vincristine, and prednisone did not alter the transfusion requirement. The studies reported here were performed prior to and after the patient had received blood transfusions. Informed consent was obtained from both the patient and normal donors.

MATERIALS AND METHODS

Cell surface analysis of leukemic cells. Peripheral blood lymphocytes were separated from heparinized whole blood by Ficoll-sodium metrizoate gradient centrifugation.6 The number of thymus-derived (T) lymphocytes was determined by the spontaneous cold sheep red blood cell (SRBC) rosette method as described by Ross et al.2 The number of B lymphocytes was assayed by enumerating the number of surface Ig (Slg)-bearing cells as described by Preud'homme et al.4 Lymphocytes were incubated with fluorescein-conjugated polyvalent or monospecific (IgM, IgG, IgA) rabbit or goat anti-human Ig and mounted on glass slides. The percentage of Slg-bearing cells was determined using epillumination with a Leitz ultraviolet microscope.

The percentage of (T) bone marrow cells was determined by the method of Kagan et al.8 Bone marrow cells (50 μl) at 3 × 10⁶ cells/ml were mixed with 50 μl washed SRBC at a concentration of 2 × 10⁸ cells/ml and 25 μl heat-inactivated SRBC-adsorbed fetal calf serum. This mixture was incubated for 5 min at 37°C, centrifuged at 200 g for 3 min at 24°C, and then incubated for 3-6 hr at 4°C. Four fields of 100 cells were counted in a hemocytometer, and cells were scored as positive if they bound four or more SRBC. 6 × 10⁵ bone marrow rosette-forming cells were resuspended in NCTC-109 (Microbiological Associates, Walkersville, Md); 94% of the cells in this final suspension were capable of forming SRBC rosettes. The SRBC were lysed with a 1% ammonium chloride solution.

Anti-thymocyte globulin (ATG) treatment. 4 × 10⁶ peripheral blood lymphocytes from the patient with T cell CLL were incubated for 60 min at 37°C in 3% CO₂ with and without 0.5 ml of a 1:1000 dilution of ATG (Upjohn, lot 161,388) and 0.05 ml of a 1:2 dilution of pooled fresh human serum as a source of complement. Following incubation the cells were washed three times, and viability was assayed using a 0.1% trypan blue solution. The patient's bone marrow cells and those of a normal control were treated with ATG in a similar fashion.

CFU-E assay. The plasma clot culture technique for measuring in vitro the growth of erythroid colonies from human marrow has been described by Tepperman et al.9 Dispersed bone marrow cells at a final concentration of 6 × 10⁵ cells per 1.1 ml were cultured in duplicate in the presence or absence of 2 IU erythropoietin. In studies of humoral inhibitors 0.05 ml Millipore-filtered sera was added. In the peripheral blood lymphocyte studies 4 × 10⁵ lymphocytes from
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our patient, three other patients with B cell CLL, and ten normal controls were cocultured with $6 \times 10^5$ normal bone marrow cells. $4 \times 10^3$ of the patient’s bone marrow-derived T lymphocytes both prior to and following ATG treatment were also cocultured with normal human marrow.

The ability of the patient’s own marrow to generate erythroid colonies in the presence or absence of 2 IU erythropoietin was determined. The influence of prior ATG treatment on erythroid colony growth in marrow obtained from the patient and from a normal control was also assessed.

Cultures were maintained in a humidified atmosphere of 3% CO$_2$ in air at 37°C. After 7 days of incubation the clots were removed and transferred to glass slides, fixed in a glutaraldehyde, and stained with benzidine and hematoxylin. Under 100x magnification each clot was examined, and erythroid colonies consisting of eight or more benzidine-positive cells were counted. Lymphocytes from both the patient and at least two normal donors were studied simultaneously, the same bone marrow donor being used.

RESULTS

On four separate occasions 95% of the patient’s peripheral blood lymphocytes formed spontaneous rosettes with SRBC, while 2% of the cells had demonstrable Slg. In our laboratory 72% of normal human blood mononuclear cells form SRBC rosettes, while 24% are Ig bearing. Of the patient’s bone marrow cells, 56% separated by Ficoll-sodium metrizoate centrifugation formed spontaneous SRBC rosettes, while 19% of normal human marrow cells are capable of rosetting.

Humoral inhibitors of erythropoiesis were sought by the culture of normal human bone marrow in the presence of serum from our patient with T cell CLL. The plasma clot assay system for erythroid colonies requires erythropoietin in order for normal human bone marrow to form erythroid colonies. No erythroid colonies developed in the absence of erythropoietin. Addition of sera from normal humans or from the patient did not influence colony formation $(275 \pm 26$ versus $279 \pm 22$ and $273 \pm 30$ colonies/$6 \times 10^5$ cells). By contrast, serum from a patient with pure red cell aplasia (as had also been previously shown$^{10}$) used as positive control in the present study inhibited the formation of erythroid colonies by normal bone marrow $(275 \pm 26$ versus $42 \pm 8$ colonies/$6 \times 10^5$ cells). These results suggest that a serum inhibitor of erythropoiesis was not instrumental in the development of the anemia in this patient.

The effect of peripheral blood lymphocytes on erythroid colony formation by normal human bone marrow is shown in Table 1. When the patient’s peripheral blood lymphocytes were cocultured with normal marrow, significant suppression of erythroid colony formation was observed. Lymphocytes from normal subjects and from three patients with B cell CLL did not influence erythroid colony formation.

Table 2 shows the results of replicate cultures involving cocultivation of the patient’s bone marrow-derived T cells with normal human bone marrow (single donor). While the number of erythroid colonies produced by this donor’s marrow was lower than usual, the results shown in Table 2 clearly show that the patient’s marrow lymphocytes significantly suppressed erythroid colony formation by this bone marrow. However, when these lymphocytes were pretreated with ATG and pooled human sera, suppression was no longer observed. Similar results were obtained with other bone marrow donors (with normal proliferative activity in vitro). However, in these latter studies the patient had
Table 1. Effect of Peripheral Blood Lymphocytes From a Patient With T Cell CLL, Normal Donors, and Patients With B Cell CLL on Erythroid Colony Formation by Normal Human Bone Marrow Cells In Vitro

<table>
<thead>
<tr>
<th>Additions to Culture</th>
<th>No. of Erythroid Colonies/6 x 10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>272 ± 38</td>
</tr>
<tr>
<td>4 x 10^5 normal lymphocytes</td>
<td>276 ± 12</td>
</tr>
<tr>
<td>4 x 10^5 T cell CLL lymphocytes*</td>
<td>128 ± 19</td>
</tr>
<tr>
<td>4 x 10^5 B-Cell CLL lymphocytes</td>
<td>288 ± 36</td>
</tr>
</tbody>
</table>

All cultures contained 2 IU erythropoietin. Each value represents the mean ± 1 SE of results obtained from replicate cultures. Similar results were obtained in five additional studies, two prior to and three after the patient had received blood transfusions.

*Obtained prior to transfusion.

Table 2. Effect of T Lymphocytes Obtained From Bone Marrow of a Patient With T Cell CLL on Erythroid Colony Formation by Normal Human Bone Marrow Cells In Vitro

<table>
<thead>
<tr>
<th>Additions to Cultures</th>
<th>No. of Erythroid Colonies/6 x 10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>176 ± 36</td>
</tr>
<tr>
<td>4 x 10^5 marrow T lymphocytes</td>
<td>39 ± 15</td>
</tr>
<tr>
<td>4 x 10^5 marrow T lymphocytes pretreated with ATG and complement</td>
<td>144 ± 12</td>
</tr>
</tbody>
</table>

All cultures contained 2 IU erythropoietin. Bone marrow aspirates were obtained from the patient prior to transfusion. Each value represents the mean ± 1 SE of replicate cultures. Similar results were obtained in three additional studies conducted with posttransfusion bone marrow aspirates.

received several blood transfusions. Since we are limiting the presentation of data to pretransfusion studies, these results are not included in the table.

As shown in Table 3, the patient's own bone marrow cells were incapable of forming erythroid colonies in the presence or absence of erythropoietin. Moreover, prior treatment of these cells with ATG and pooled human sera resulted in a significant enhancement of erythroid colony formation in the presence of erythropoietin. The ability of normal human marrow cells to generate erythroid colonies was not influenced by ATG pretreatment (Table 3).

DISCUSSION

In the present study we have attempted to clarify the mechanism of the erythropoietic failure in a patient with advanced T cell CLL.

Early investigations suggested that the degree of anemia in neoplastic disorders paralleled the extent of tumor invasion of the marrow,11,12 i.e., that the decreased production of red blood cells occurred secondary to a crowding out

Table 3. Erythroid Colony Formation by Bone Marrow Cells Obtained From a Patient with T Cell CLL Prior to Transfusion Therapy and a Normal Control in Response to Erythropoietin In Vitro

<table>
<thead>
<tr>
<th>Source of Bone Marrow</th>
<th>Prior Treatment of Bone Marrow Cells</th>
<th>No. of Erythroid Colonies/6 x 10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal donor</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Normal donor</td>
<td>Complement alone</td>
<td>272 ± 38</td>
</tr>
<tr>
<td>Normal donor</td>
<td>ATG + Complement</td>
<td>242 ± 32</td>
</tr>
<tr>
<td>T cell CLL</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>T cell CLL</td>
<td>Complement alone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ATG + Complement</td>
<td>84 ± 17</td>
</tr>
</tbody>
</table>

Each value represents the mean ± 1 SE of results obtained from replicate cultures. Similar results were obtained in two additional studies with patient's bone marrow obtained after transfusion therapy had been instituted.
of hematopoietic stem cells by malignant cells. Subsequent studies have failed to confirm the correlation between the volume of marrow replaced by tumor cells and the severity of anemia.\textsuperscript{13}

Zucker et al.\textsuperscript{14} used marrow culture procedure in vitro to measure the effect of erythropoietin on heme synthesis in a group of patients with anemia associated with neoplasma. In 9 of 11 patients with more than 60\% of the marrow replaced by malignant cells, no response to erythropoietin was noted. The mechanism by which malignant cells blunted the effect of erythropoietin remains unknown.

Utilizing a culture system more conducive to the proliferation and differentiation of erythropoietin-responsive stem cells, we were able to better define the nature of the anemia associated with advanced malignancy. The marrow cells of our patient were incapable of showing erythroid proliferation in vitro in the presence or absence of erythropoietin. However, after the cells were treated with ATG and complement, colony formation was dramatically enhanced. It seems likely that ATG inactivated or destroyed a population of cells capable of interfering with the usual proliferative and/or differentiative response of the erythroid-committed stem cell to erythropoietin and thus permitted these cells to respond to the hormone. It should be noted, however, that the numbers of colonies produced by the patient's marrow after treatment with ATG, while highly significant, did not approach the normal levels. Whether or not this implies that not all the suppressive elements were neutralized by treatment with the ATG remains to be determined.

These results indicate that the anemia in this patient cannot be ascribed entirely to an absolute deficiency of hematopoietic stem cells, since we showed that a population of lymphoid cells may have been responsible for the suppression of erythropoiesis in this individual. The presence of these cells in this patient was not associated with transfusion therapy. The results presented in this report were obtained before the patient received any transfusion. No significant difference in the suppressor activity of the patient's T cells was noted following the institution of transfusion therapy. It is also unlikely that this suppressor activity can be attributed to HLA incompatibility, since suppressor activity was directed against both heterologous and autologous bone marrow erythroid precursors.

It is possible that a similar relationship between neoplastic cells and hematopoietic stem cells accounts for the cytopenia associated with other advanced malignancies. However, such a relationship could not be shown in three patients with typical B cell CLL.

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