Pure Red Cell Aplasia and Hypogammaglobulinemia Associated With Tr-Cell Chronic Lymphocytic Leukemia

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A 72-yr-old male with Tr-cell chronic lymphocytic leukemia (Tr-CLL) exhibited pure red cell aplasia (PRCA) and hypogammaglobulinemia. During a remission of Tr-CLL, and while receiving cyclophosphamide therapy, he recovered from PRCA and hypogammaglobulinemia. To investigate the pathogenesis of PRCA and hypogammaglobulinemia, we used coculture techniques to study the effect of the malignant Tr cells on erythroid colony formation and B-cell differentiation to immunoglobulin-producing cells. Varying numbers of malignant Tr cells (2 × 10^4 to 2 × 10^6 cells) were cocultured with 2 × 10^5 normal bone marrow cells. The malignant Tr cells caused a marked reduction of erythroid colony formation in the plasma clot system. This suppression of erythroid colony formation was reversed when the malignant Tr cells were pretreated with antilymphocyte serum and complement. There was no evidence of inhibitory effects in the serum or the supernatant media of the malignant Tr cells stimulated with phytohemagglutinin (PHA). The malignant Tr cells, stored at −80°C before transfusion, were also capable of suppressing autologous erythroid colony formation after recovery from PRCA. In addition, malignant Tr cells were found to have strong suppressor activity against the immunoglobulin biosynthesis by allogeneic B cells. The in vitro suppressions of both erythroid colony formation and B-cell differentiation provide an explanation for the association of PRCA and hypogammaglobulinemia with Tr-CLL.

C H R O N I C lymphocytic leukemia (CLL) is usually derived from a malignant proliferation of a B-cell line, however, on occasion, it has been identified as bearing the T-cell line. The anemia in CLL has been attributed to reduced red cell production capacity and/or shortened red cell survival.

Dameshek and colleagues described severe erythroid aplasia and reticulocytopenia in CLL. In 1974, Abeloff and Waterbury reported a case of CLL with pure red cell aplasia (PRCA) that experienced remission of both PRCA and CLL while undergoing cyclophosphamide therapy.

In this article we describe a case of Tr cell (T cell with Fc receptors for IgG) CLL complicated with PRCA and hypogammaglobulinemia. In the present study we have attempted to investigate the effect of the malignant Tr cells on erythroid colony formation and immunoglobulin production by using in vitro coculture techniques.

CASE REPORT

The patient, a 72-yr-old Japanese male, was referred to the Hematology Division of Tsukuba University Hospital in July 1978 for evaluation of severe anemia. Physical examination on admission revealed marked pallor but no palpable lymphadenopathy and hepatosplenomegaly. There were no signs of erythroderma or skin nodes.

Chest x-ray film showed no hilar lymphadenopathy or thymoma. Blood cell count revealed the following: RBC 1.21 × 10^12/liter, Hb 3.7 g/dl, Hct 10.9%, platelet count 492 × 10^9/liter, reticulocytes 0.2%, WBC 12.1 × 10^9/liter with 2% band forms, 4% segmented neutrophils, 6% monocytes, and 88% mature lymphocytes. The lymphocytes in the peripheral blood were small, uniform, and mature and had a high nuclear/cytoplasmic ratio. Many smudge cells were seen in the smear. Electron microscopy of these cells (Fig. 1) showed round or oval nuclei with dispersed heterochromatin, which were clearly distinct from those of Sézary cells. There were abundant mitochondria, moderately developed Golgi apparatus, strands of rough endoplasmic reticulum (RER), and structures of parallel tubular array in the cytoplasm. The bone marrow aspirate showed normocellularity with diffuse infiltration of mature lymphocytes (61% of bone marrow cells) and a lack of erythroid precursors (2% of bone marrow cells). The granulocyte precursors were normal in number with orderly maturation. Numerous megakaryocytes were seen. The number of erythroid colony-forming units (CFU-E) was markedly decreased to 10.5 × 2.4/2 × 10^5 cells (control: 174.3 × 21.4/2 × 10^5 cells). However, the number of colony-forming units in culture (CFU-C) was normal.

The serum iron value was 198 μg/dl and the total iron-binding capacity was 226 μg/dl. The ferrokinetic studies showed decreased plasma iron clearance (PIT, T1/2: 145 min) and 40.3% incorporation at 2 wk. Serum erythropoietin level, as determined by a polycythemic mouse assay, was very high (1 U/ml).

The determination of serum immunoglobulin level showed IgG 540 mg/dl, IgA 162 mg/dl, and IgM 230 mg/dl. No M-bow was detected in the immunoelectrophoresis. Direct and indirect Coombs tests were negative. The activity of terminal deoxynucleotidyl transferase of the peripheral blood lymphocytes was absent.

The surface markers and functions of the peripheral lymphocytes are shown in Table 1. From July 1978 to February 1979, the patient required 8–9 U (1 U: 100 ml of packed red cells) of blood transfusions monthly to maintain his hemoglobin level. Cyclophosphamide (100 mg/day p.o.) was started on March 1, 1979. Four weeks after beginning cyclophosphamide therapy, the patient exhibited reticulocytosis, a rise in hematocrit value, and decreased lymphocytes in the peripheral blood. The bone marrow aspirate at this time showed normocellularity, with an adequate number of erythroid precursors (36.0% of
bone marrow cells), and lymphocytes were decreased to 12.8% of bone marrow cells. The number of CFU-E after the recovery from PRCA increased to $164 \times 18.6/2 \times 10^3$ cells. Cyclophosphamide therapy was discontinued on March 29, 1979. The Hb level has remained around 12-13 g/dl without transfusion, and the WBC has been stable at approximately 4-5 x 10^9/liter until the present time (March 1980), although a few abnormal lymphocytes can still be seen in the smear. Following chemotherapy, the serum IgG level has also been elevated from 540 mg/dl to 1880 mg/dl. Figure 2 shows the clinical course in detail.

MATERIALS AND METHODS

Isolation of Peripheral Blood Lymphocytes

Peripheral blood lymphocytes obtained from the patient and normal donors were separated from freshly drawn heparinized blood by Ficoll-Hypaque density sedimentation.7

Characterization of the Peripheral Blood Cells

T-cell determination was carried out by a spontaneous cold sheep red cells assay,8 and B-cells were evaluated by the immunofluores-
cent technique for surface immunoglobulin. Receptors for complement were detected by rosette formation with complement-coated zymosan as described by Huber and Wigzell. Receptors for the Fc portion of IgG and IgM were detected by rosette formation with ox red blood cells (ORBC), which were coated with purified rabbit IgG and IgM anti-ox red blood cells.

**Preparation of Normal T Cells**

A T-cell-rich fraction from normal donors was obtained by E-rosette centrifugation through Ficoll-Hypaque, and lysis of an attached E rosette was performed by the addition of 0.83% Tris-buffered ammonium chloride. By using these procedures, a suspension containing more than 95% T cells, as tested by the E-rosette method, was obtained.

**Phytohemagglutinin (PHA) Responsiveness In Vitro**

³H-thymidine uptake of the peripheral blood lymphocytes was measured in the presence of PHA-P (Gibco, Grand Island, N.Y.) according to the method described by Hughes and Caspary. The activity of the peripheral blood lymphocytes was assayed using the method described by McCaffrey et al.

**Assay for Helper or Suppressor Function**

Assay methods for helper or suppressor function were followed as described elsewhere. Briefly, the mononuclear cells from normal donors were separated from heparinized blood bi Ficoll-Hypaque density centrifugation. Adherent cells were removed by plastic dish adherence. Nonadherent cells were separated into a T-cell-rich fraction (Tₜ) and a B-cell-rich fraction (Bₜ) by the E-rosette centrifugation method described above. The T-cell fraction from the patient (Tₚ) was separated in the same manner. The number of cells in Tₜ, Bₜ, and Tₚ were counted. Preparations of 2 x 10⁶ Bₜ, 8 x 10⁷ Tₜ, and 2 x 10⁸ Tₚ were cultured in the presence of 10 μg/ml of pokeweed mitogen (PWM) (Gibco, Grand Island, N.Y.) in 5% CO₂ for 7 days. Total volume of each culture was adjusted to 1 ml. The number of cytotoxic immunoglobulin-positive cells (CIg-positive cells) in each culture was determined as described previously. The cytotoxic immunoglobulin-positive cells were well generated in the presence of PWM when 2 x 10⁸ cells were cultured with 8 x 10⁶ autologous T cells. The formula for helper function was as follows:

\[
\text{Helper index} = \frac{\text{CIg-positive cells in } (Bₜ + Tₚ)}{\text{CIg-positive cells in } Bₜ} \times 100
\]

When Tₚ cells had helper effects, the helper index was close to 100 (normal values: 100 ± 20). The formula for suppressor function was as follows:

\[
\text{Suppressor index} = \left(1 - \frac{\text{CIg-positive cells in } (Bₜ + Tₚ + Tₚ)}{\text{CIg-positive cells in } (Bₜ + Tₚ)}\right) \times 100
\]

When Tₚ had strong suppressive effects, the suppressor index was close to 100 (normal values: 0 ± 45).

**Cytotoxicity Assays**

Cytotoxicity cultures in microplates employing ⁵¹Cr-labeled chicken red blood cells as target cells were carried out as described by Saksela and Häyry. The system consisted of effector cells, target cells, and (1) medium alone for spontaneous cell-mediated cytotoxicity (SCMC, NK-cell activity), (2) PHA-P or concanavalin A (Con-A) (Gibco) at a final concentration of 2 μg/ml for mitogen-induced cellular cytotoxicity (MICC, cytotoxic T-cell activity), or (3) rabbit anti-chicken red blood cell serum at a final dilution of 2 x 10⁻⁵ for antibody-dependent cellular cytotoxicity (ADCC, K-cell activity). Target cells incubated in medium alone without added effector cells were included in each experiment for the determination of the percentage of spontaneous ⁵¹Cr release. Target cell aliquots were frozen and thawed for the determination of the maximum percentage of ⁵¹Cr releasable. Target cell damage manifested by ⁵¹Cr release was expressed as the percentage of ⁵¹Cr release, as described elsewhere, or as the percentage of lysis using the following formula:

\[
\text{Percent lysis} = \frac{\text{Experimental } % \text{ ⁵¹Cr release} - \text{Spontaneous } % \text{ ⁵¹Cr release}}{\text{Maximal } % \text{ ⁵¹Cr release} - \text{Spontaneous } % \text{ ⁵¹Cr release}} \times 100
\]

(Experimental % ⁵¹Cr release = % ⁵¹Cr release due to SCMC, MICC, or ADCC cultures).

**Freezing Procedure of Peripheral Blood Lymphocytes**

Lymphocytes were isolated as described above, and were frozen using a programmed freezing apparatus (Cryo-Med, Mt. Clemens, Mich.). Frozen cells were kept at -80°C in a concentration of 2 x 10⁹/ml in RPMI 1640 supplemented with 15% fetal calf serum (FCS) (Flow Laboratories, Rockland, Md.) and 10% dimethylsulfoxide (DMSO) (Merk Parrmst, West Germany) for later experiments. When needed for further experiments, the cells were thawed and washed twice to remove DMSO, then checked for viability with Trypan blue.

**Antilymphocyte Serum (ALS) Treatment**

A sample of 2 x 10⁶ peripheral blood lymphocytes from the patient were incubated for 60 min at 37°C in 5% CO₂ with 0.5 ml of a 1:500 dilution of ALS (Midori, Japan) and 0.5 ml of a 1:2 dilution of fresh human serum. After the incubation, the cells were washed three times and their viability was assayed using a 0.1% Trypan blue solution.

**Preparation of PHA-Stimulated Lymphocyte Conditioned Medium**

Lymphocytes conditioned medium stimulated by PHA-P was prepared from peripheral lymphocytes of normal donors and the patient. Lymphocytes were separated through Ficoll-Hypaque and then washed three times with RPMI 1640 supplemented with 5% FCS. These cells were resuspended in RPMI 1640 supplemented with 15% FCS in a concentration of 2 x 10⁶/ml. The PHA-P was added in a concentration of 1.0% (v/v). After incubation for 24 hr at 37°C, 5% CO₂ humidified incubator, the cells were washed to remove the excess of PHA-P and resuspended in the same medium without PHA-P in a concentration of 2 x 10⁶/ml. After incubation for 6 days, the cells were removed by centrifugation, and the supernatant was stored at -20°C until use.

**CFU-E and CFU-C Assay**

CFU-E assay was carried out by plating 2 x 10⁶ bone marrow cells according to the method described by Tepperman et al. Cell aggregates with more than 8 positive benzidine-stained cells were considered a colony. CFU-C assay was performed by plating 2 x 10⁶
bone marrow cells using the method of Pike and Robinson. Cell aggregates containing greater than 40 cells were counted by inverted microscope as a colony.

**Coculture Studies**

Normal bone marrow cells from ABO compatible donors were used for all coculture studies. In the lymphocyte studies, varying numbers (2 x 10^2, 2 x 10^3, 2 x 10^4, and 2 x 10^5) of patient’s lymphocytes, 2 x 10^5 patient’s lymphocytes pretreated with ALS and complement, and 2 x 10^5 normal T cells were cocultured with 2 x 10^5 normal bone marrow cells to assay CFU-C and CFU-E. To study humoral inhibitors, 0.1 ml of lymphocyte conditioned medium from normal donors and the patient and 0.1 ml of serum from normal donors and the patient were mixed with 2 x 10^5 normal bone marrow cells, and then CFU-E and CFU-C were assayed. After recovery of erythropoiesis, 2 x 10^5 of the patient’s lymphocytes frozen before blood transfusion were cocultured with 2 x 10^5 autologous bone marrow cells.

**RESULTS**

**Immunologic Studies**

Table 1 lists the cell identification and function of the peripheral blood lymphocytes. The peripheral blood lymphocytes showed that 99.5% of the cells form E rosettes, 94.0% of the cells bear Fc receptors for IgG, 3.5% of the cells have complement, and 0.75% of the cells have membrane-bound immunoglobulin. Based on these results, the peripheral blood lymphocytes were identified as Tr cells (T cells with Fc receptors for IgG). These T cells exhibited K-cell activity (ADCC) and also cytotoxic T-cell activity induced by Con-A. The response of peripheral blood lymphocytes to PHA-P was only 0.11% of control. The tuberculin test, PHA skin test, and dinitrochlorobenzene (DNCB) skin test were negative, indicating severe impairment of T-cell function in vitro and in vivo.

**The Effect of Malignant Tr Cells on Immunoglobulin Production**

The effect of malignant Tr cells on PWM-induced B-cell differentiation is reported in Table 2. The suppressor index for normal T cells was 0 ± 45 in our laboratory control. In our experiments, it was found that B cells failed to differentiate to immunoglobulin-producing cells in the presence of PWM when 4 x 10^5 malignant Tr cells were added to this system. The suppressor index of malignant Tr cells was 89.5. No helper effect on immunoglobulin production was found when 8 x 10^3 malignant Tr cells were added to 2 x 10^5 normal B cells.

**The Effect of Malignant Tr Cells on Erythroid Colony Formation**

The effect of malignant Tr cells on erythroid colony formation of normal bone marrow cells is demonstrated in Table 3. When 2 x 10^5 malignant Tr cells were cocultured with 2 x 10^5 normal bone marrow cells, the number of CFU-E was markedly decreased to 8.5 ± 4.5/2 x 10^5 cells (control: 174.3 ± 21.4/2 x 10^5 cells). This suppression of allogeneic erythroid colony formation was almost reversed when malignant Tr cells were pretreated with ALS and complement. When the malignant Tr cells added to the culture system were varied from 2 x 10 to 2 x 10^5, the CFU-E was proportionally suppressed. In order to study the effect of malignant Tr cells on autologous bone marrow cells, 2 x 10^5 malignant Tr cells frozen before transfusion were cocultured with 2 x 10^5 autologous bone marrow cells after the recovery of erythropoiesis, and the number of CFU-E in this coculture system was also decreased to 12.4 ± 4.4/2 x 10^5 cells. On the other hand, no suppression of CFU-E was found when malignant Tr cells, serum, and the supernatant of PHA-stimulated lymphocytes were added to coculture system.

Humoral inhibitors influencing erythropoiesis in the serum or the supernatant media of the malignant Tr cells stimulated with PHA-P were investigated. When 0.1 ml of serum was added to the culture system, the erythroid colony formation of normal bone marrow cells was not suppressed, indicating no evidence of the inhibitor in the serum. Furthermore, no inhibitory effects were found in the supernatant media of the malignant Tr cells stimulated with PHA-P.

Our results in coculture experiments indicate that
Table 3. Effect of the Malignant Tr Cells, Serum From the Patient, and the Supernatant of PHA-Stimulated Malignant Tr Cells on Erythroid Colony Formation by Normal Bone Marrow Cells In Vitro

<table>
<thead>
<tr>
<th>Addition to Culture</th>
<th>Source of Bone Marrow</th>
<th>No. of CFU-E/2 x 10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Normal donor</td>
<td>174.3 ± 21.4</td>
</tr>
<tr>
<td>2 x 10⁶ malignant Tr cells</td>
<td>Normal donor</td>
<td>132.5 ± 40.6</td>
</tr>
<tr>
<td>2 x 10⁷ malignant Tr cells</td>
<td>Normal donor</td>
<td>122.5 ± 36.2</td>
</tr>
<tr>
<td>2 x 10⁸ malignant Tr cells</td>
<td>Normal donor</td>
<td>71.4 ± 24.9</td>
</tr>
<tr>
<td>2 x 10⁹ malignant Tr cells</td>
<td>Normal donor</td>
<td>55.0 ± 10.4</td>
</tr>
<tr>
<td>2 x 10⁹ cells pretreated with ALS and complement</td>
<td>Normal donor</td>
<td>8.5 ± 4.5</td>
</tr>
<tr>
<td>2 x 10⁹ normal T cells</td>
<td>Normal donor</td>
<td>163.6 ± 18.6</td>
</tr>
<tr>
<td>0.1 ml of patient’s serum</td>
<td>Normal donor</td>
<td>210.0 ± 16.6</td>
</tr>
<tr>
<td>0.1 ml of normal serum</td>
<td>Normal donor</td>
<td>194.1 ± 20.5</td>
</tr>
<tr>
<td>0.1 ml of supernatant media of PHA-stimulated malignant Tr cells</td>
<td>Normal donor</td>
<td>176.5 ± 19.3</td>
</tr>
<tr>
<td>0.1 ml of supernatant media of PHA-stimulated normal T cells</td>
<td>Normal donor</td>
<td>161.7 ± 11.9</td>
</tr>
<tr>
<td>2 x 10⁹ malignant Tr cells frozen prior to treatment</td>
<td>Patient’s bone marrow after the recovery from PRCA</td>
<td>12.4 ± 4.4</td>
</tr>
</tbody>
</table>

All coculture experiments were performed in the presence of 2 IU erythropoietin.

Each value represents mean ± 1 SD (n = 5).

The cultures were carried out for 7 days at 37°C in a humidified atmosphere of 5% CO₂ in air.

the malignant Tr cells in CLL could act on both lymphocytic and hematopoietic target cells.

Clinical Course

As shown in Fig. 2, this patient had a remission of CLL and recovered from pure red cell aplasia and hypogammaglobulinemia after cyclophosphamide therapy. The clinical course suggests that there is a close relation between the decreased number of malignant Tr cells and recovery from PRCA and hypogammaglobulinemia. The surface markers of peripheral blood lymphocytes during CLL remission showed 80.5% of cells to form E rosettes, 56.5% to bear Fc receptors for IgG, 15.5% to have receptors for complements, and 16.5% to have membrane-bound immunoglobulin. The response of peripheral blood lymphocytes to PHA-P was 45.6% of control. The tuberculin test and PHA skin test were weakly positive. This case has been in remission for 1 yr without therapy.

DISCUSSION

The previous report of CLL associated with PRCA emphasized the rarity of its occurrence. The mechanism of complication of PRCA with CLL has not been understood.

Recently, using coculture techniques, Hoffman et al. suggested that an interaction between the malignant T cell and erythropoietin-responsive stem cell was important in the development of anemia in T-cell CLL. We described a rare case of Tr-cell CLL complicated by PRCA and hypogammaglobulinemia. In this report, we have attempted to investigate an in vitro interaction among erythroid colony-forming cells, immunoglobulin-producing cells, and the malignant Tr cells. The CFU-E was strongly suppressed when the malignant Tr cells were cocultured with normal allogeneic bone marrow cells. However, after the cells were treated with ALS and complement, CFU-E was dramatically reversed. The malignant Tr cells frozen prior to treatment of the patient also suppressed CFU-E of autologous bone marrow cells after the recovery from PRCA. The suppression of autologous CFU-E was also reversed when the malignant Tr cells were pretreated with ALS and complement. This is a direct demonstration that the malignant Tr cells are capable of suppressing autologous hematopoietic stem cells. In contrast, erythroid colony formation was significantly increased when normal T cells were cocultured. Subsequent investigations have failed to demonstrate inhibitory effects in the serum or the supernatant media of lymphocytes cultured with PHA-P. Our preliminary experiments showed that freezing and thawing of the malignant Tr cells removed their suppressive effects on erythroid colony formation, suggesting the need for cellular integrity. These results indicate that the cell-to-cell interaction between the malignant Tr cell and hematopoietic stem cell may be very important for the mechanism of erythroid colony suppression in vitro.

On the other hand, it is known that human T lymphocytes can express surface Fc receptors for IgG (Tα cells) and IgM (Tµ cells). In this paper, T cells with Fc receptors for IgG are referred to as Tr cells and those with IgM receptors as Tµ cells.) It is also known that Tr and Tµ cells have different morphological characteristics and remarkable functional differences on B-cell proliferation into plasma cells in response to PWM stimulation. In 1977, Moretta et al. stated that Tr cells suppressed the differentiation of plasma cells, whereas Tµ cells helped it. However, in 1978, Hayward et al. reported that concanavalin A stimulated Tµ cells to suppress the PWM-induced differentiation of B lymphocytes to
plasma cells, suggesting heterogeneity of human suppressor T cells.22

These malignant cells exhibited surface Fc receptors for IgG, referred to as malignant Tr cells, and showed suppressor activity against immunoglobulin biosynthesis by allogeneic B cells. Our results obtained from in vitro coculture studies suggest that the malignant Tr cells have suppressor activity against both erythroid cell and B-cell differentiation, thereby causing pure red cell aplasia and hypogammaglobulinemia in vivo. Our patient recovered from both PRCA and hypogammaglobulinemia during a remission of CLL induced by cyclophosphamide therapy. The clinical course suggests that the malignant Tr cells may have played an important role in the pathogenesis of PRCA and hypogammaglobulinemia. Furthermore, these clinical findings are consistent with the results obtained from in vitro coculture studies.

Suppressor cells against erythropoiesis have been found in cases of Diamond-Blackfan's syndrome24 and aplastic anemia.25 On the other hand, suppressor cells against granulopoiesis have been demonstrated in a case of Felty's syndrome.26 Suppressor cells against immunoglobulin synthesis in vitro have also been found in a case of multiple myeloma.27 Hodgkin's disease,28 variable immunodeficiency,29 and aplastic anemia.30 Lymphocytes rarely suppress both hematopoietic stem cells and immunoglobulin-producing cells simultaneously. Lilwin and Zanjani31 reported that lymphocytes from two patients with immunodeficiency and thymoma suppressed both immunoglobulin production and erythroid differentiation simultaneously. However, there was no evidence that these suppressor cells were derived from a clonal origin. In contrast, it is interesting that clonal malignant Tr cells suppressed both immunoglobulin production and erythroid differentiation in the present case.

It is possible that the suppressor activity might be attributed to HLA incompatibility due to blood transfusion. To avoid this possibility, the malignant Tr cells were frozen and stored at –80°C before blood transfusion and used for the later studies. It was confirmed that these stored malignant Tr cells suppressed autologous erythroid colony formation after the recovery of PRCA.

We conclude that malignant Tr cells are capable of suppressing erythropoiesis and immunoglobulin production in vitro. This may explain the association of pure red cell aplasia and hypogammaglobulinemia with Tr-CLL.

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