T Gamma (Tγ) Cells Suppress Growth of Erythroid Colony-forming Units In Vitro in the Pure Red Cell Aplasia of B-Cell Chronic Lymphocytic Leukemia

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ABSTRACT In vitro studies were performed in two patients with B-cell chronic lymphocytic leukemia who developed pure red cell aplasia (CLL-PRCA). During the active phase of their red cell aplasia, there was a marked reduction in the numbers of erythroid colony-forming units (CFU-E). Unfractionated sera or separated IgG fractions from these patients did not impair CFU-E proliferation from either autologous or allogeneic marrows. Increased numbers of T lymphocytes were present in marrow aspirates of these patients. Analysis of these T cells indicated that 90 and 35%, respectively, bore Fc receptors for IgG (Tγ cells). Removal of T cells by E-rosetting techniques augmented CFU-E growth in CLL-PRCA 10-fold. Similar treatment of normal marrows did not cause similar enhanced growth of CFU-E. Co-cultures of marrow T cells or Tγ cells obtained during the active phase of CLL-PRCA suppressed CFU-E growth from autologous or allogeneic marrows. After achieving drug-induced remission of the PRCA, marrow T cells were no longer inhibitory. In contrast, BFU-E (erythroid burst-forming units) or granulocyte proliferation in diffusion chambers were not suppressed by CLL-PRCA T cells. These findings suggest that the development of PRCA in B-cell CLL may result from suppression of CFU-E proliferation by Tγ cells.

INTRODUCTION

The majority of patients with B-cell chronic lymphocytic leukemia (CLL)[1] develop anemia sometime during the course of their disease. In 10 to 20% of these patients, the anemia results from an autoimmune hemolytic process (1). However, in most patients, the anemia of CLL is due to decreased red cell production (2, 3). An extreme manifestation of this production defect is the complete cessation of red cell production (pure red cell aplasia, PRCA), a syndrome noted in a small number of CLL patients (4). This latter group have provided an opportunity to examine the operative mechanism(s) for the anemia associated with this leukemia. Previous studies indicated that serum IgG inhibitors are not responsible for the PRCA in CLL (5, 6); by contrast, these can account for up to 50% of idiopathic PRCA (5–7).

Hoffman et al. (8) recently found that T cells from a patient with a T-cell variant of CLL suppressed proliferation of the mature erythroid colony-forming cells (CFU-E). Nagasawa et al. (9) subsequently showed that the malignant T cells bearing Fc receptors for IgG, termed T gamma (Tγ) cells, suppressed both erythroid colony formation and B-cell differentiation in

Portions of this work were presented at the 23rd Annual Meeting of the American Society of Hematology, San Antonio, TX, December 1981.

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Received for publication 9 March 1982 and in revised form 19 August 1982.

[1] *Abbreviations used in this paper*: BFU-E, erythroid burst-forming units; CFU-E, erythroid colony-forming units; CLL, chronic lymphocytic leukemia; CVP, cyclophosphamide, vincristine, and prednisone; DC, diffusion chamber; E-rosetting, sheep erythrocyte rosetting; FH, Ficoll-Hypaque; Hb, hemoglobin; HCT, hematocrit; LDMNC, light density mononuclear cells; α-MEM, alpha minimal essential medium; PRCA, pure red cell aplasia; TD-LDMNC, T-cell-depleted LDMNC; WBC, leukocyte(s).

vitro. Moreover, we have shown that T cells from four patients with B-cell CLL-PRCA were defective stimulators of the primitive blood erythroid burst-forming units (BFU-E) (6). The defective burst-promoting function of these T cells was correlated with the presence of increased numbers of Tγ cells (10).

We recently had the opportunity to study the serum and cellular interactions of marrow CFU-E in two additional patients with B-cell CLL who developed PRCA. This report provides evidence for T-cell suppression of marrow erythropoiesis in B-cell CLL with PRCA. Furthermore, the suppressor cells appear to be confined to the Tγ cell subset.

METHODS

Case reports. Patient A, a 45-yr-old White female was seen in December of 1979 with complaints of profound fatigue and weakness. Physical examination revealed diffuse lymphadenopathy, splenomegaly, and absent cutaneous lesions. Laboratory examination revealed a hematocrit (HCT) of 15%, hemoglobin (Hb) 5.0 g/dl, platelets 350,000/μl, and reticulocyte 0.1%. The leukocyte (white blood cell, WBC) count was 49,000/μl with a differential count of 84% small round lymphocytes, 14% segmented neutrophils, and 2% monocytes. Direct Coombs test, serum bilirubin, haptoglobin, iron and total iron binding capacity, vitamin B_{12}, folic acid, creatinine, serum glutamic oxaloacetic transaminase (SGOT) and alkaline phosphatase were normal or negative. Serum IgG and IgM levels were decreased to 7.6 m/ml (normal 8–18) and 0.4 m/ml (normal 0.6–2.8 m/ml), respectively. Bone marrow aspirate and biopsy revealed a hypercellular marrow infiltrated with >50% small round lymphocytes. Granulocytic and megakaryocytic maturation was normal, however, no erythroid precursors could be identified. A diagnosis of B-cell CLL with PRCA (CLL-PRCA) was made. Direct immunofluorescence studies on blood lymphocytes confirmed the presence of B-cell CLL, IgM, lambda type. Nuclear morphologic findings by light and electron microscopy, sheep erythrocyte rosetting (E-rosetting) studies and acid phosphatase reaction of blood lymphocytes further excluded a diagnosis of T-cell CLL. The patient was transfused and treated for 2 mo with chemotherapy (chlorambucil, prednisone, vincristine, methotrexate, and adriamycin) without improvement of anemia. 3 wk after chemotherapy was discontinued, a repeat bone marrow aspirate and biopsy showed again, CLL-PRCA. Marrow was taken for in vitro studies. Treatment with splenic irradiation, splenectomy, and total body irradiation induced a reticulocytosis of 5.4% and the HCT rose to 42%. 6 mo later, the HCT dropped to 26.9% with 0.1% reticulocytes. The WBC count was 65,000/μl with 72% small round lymphocytes. A repeat marrow was consistent with B-cell CLL-PRCA. Treatment with oral cyclophosphamide and prednisone induced a second reticulocytosis of 4.9% after 6 wk. The HCT rose to 42% and a repeat culture study was performed off therapy. The patient has remained transfusion free for the last year, on monthly cycles of cyclophosphamide, vincristine, and prednisone (CVP).

Patient B, a 54-yr-old White female presented in January of 1976 with diffuse peripheral lymphadenopathy and mild splenomegaly. Cutaneous lesions were absent. Laboratory examination revealed a WBC count of 21,200/μl with 71% small round lymphocytes. T cell nuclear morphologic features were absent. The HCT, platelet count, and reticulocyte count were normal. A diagnosis of B-cell CLL was made. WBC rose to >100,000/μl over the ensuing months and the patient was treated with daily chlorambucil for 36 mo followed by 12 mo of pulse chlorambucil and prednisone. The therapy was discontinued. 5 mo later the patient complained of profound fatigue. The HCT was 19.3%, the WBC count 59,900/μl with 96% small round lymphocytes and 4% segmented neutrophils. Platelets were 166,000/μl. Five daily reticulocyte counts ranged between 0 and 0.1%. Direct Coombs test, serum bilirubin, haptoglobin, vitamin B_{12}, folic acid, creatinine, iron and total iron binding capacity were all normal or negative. Serum immunoglobulin levels revealed a panhypogammaglobulinemia (IgG 1.95 m/ml, IgA 0.5 m/ml, and IgM 0.13 m/ml). Bone marrow aspirate and biopsy revealed a diffuse lymphocytic infiltrate with absent erythroid precursors. Maturation of granulocytic and megakaryocytic series were normal. Direct immunofluorescence studies of blood lymphocytes confirmed the presence of B-cell CLL IgM/D lambda type. Marrow samples were taken for in vitro studies. The patient was transfused and begun on daily oral cyclophosphamide and prednisone for 1 mo followed by six monthly cycles of CVP. After 7 mo, a reticulocytosis of 5% ensued and the HCT rose to 43%. CVP was discontinued and repeat in vitro culture studies were performed. The patient remains transfusion free, off therapy 10 mo later.

Preparation of marrow or blood target cells. Marrow aspirations or venipunctures were performed on patients or normal volunteers giving informed consent, as approved by the Institutional Human Subjects Committees. Marrow aspirations were limited to 2.0 ml to avoid dilution with peripheral blood.

Light density mononuclear cells (LDMNC) or T-cell-depleted LDMNC (TD-LDMNC) retrieved from bone marrow were cultured alone or co-cultured with marrow T cells or Tγ cells in assays for marrow CFU-E. Assays for primitive BFU-E used blood null cells in co-cultures with marrow T cells or Tγ cells. In brief, marrow was aspirated into heparinized syringes diluted 1:1 with alpha minimal essential medium (α-MEM, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), strained through small bore needles and layered on a Ficoll-Hypaque (FH) gradient (specific gravity 1.077 g/cm^3). After centrifugation at 400 *g* for 30 min, whole mononuclear cells (WMNC) were retrieved from the interface and depleted of adherent monocytes by incubation on fetal calf serum-coated petri dishes (25×10^6/78 cm^2) for 1 h. The nonadherent cells referred to as LDMNC contained <5% monocytes as judged by α-napthylesterase activity and morphology. Marrow LDMNC were washed and further depleted of T cells by E-rosetting, as described below, to retrieve TD-LDMNC. The recovery of TD-LDMNC was 75% in patient A and 92% in patient B.

For BFU-E assays, blood LDMNC were further depleted of surface immunoglobulin-bearing B cells by incubation for 1 h on plastic petri dishes coated with goat anti-human immunoglobulins [$F(ab')_2$ fragment, N. L. Cappel Laboratories Inc., Cochranville, PA], as described previously (11). The B cell-depleted LDMNC were then depleted of T cells by E-rosetting, as described below for marrow T cells, to retrieve BFU-E-enriched null cell targets. These null cells contain <5% B and T cells as judged by surface immunoglobulin analysis and E-rosetting, and <2% monocytes as judged by α-napthylesterase activity.

Isolation of T cells and Tγ cells. T cells used in co-cultures were obtained by rosetting LDMNC from marrow (or

blood) with 2-aminoethylisothio uronium bromide (AET)-treated sheep erythrocytes followed by a second FH density gradient centrifugation (12). Greater than 95% of the predicted number of T cells were found in the pellets. Based on rerosetting, these T cells were >96% pure. T cells were freed of sheep erythrocytes by lysis with Tris-buffered ammonium chloride, washed with α-MEM and used in co-cultures with marrow or blood target cells.

For isolation of Tγ cells (10, 12), ox erythrocytes were sensitized with a subagglutinating titer of rabbit anti-ox erythrocyte IgG (Cappel Laboratories) to make EAIgG complexes. 3.0×10^6 T cells were then mixed with an equal volume of a 1% solution of EAIgG complexes for 30 min at 37°C, centrifuged at 200 *g* for 5 min and kept at 4°C for 1 h. The rosetted Tγ cells were then retrieved by FH density gradient centrifugation followed by lysis with Tris-buffered ammonium chloride as described above. The purity of Tγ cells were >90% when checked by rerosetting with 1% EAIgG complexes. Less than 1% of these cells were monocytes as judged by morphology and α-napthylesterase activity.

Co-culture studies and stem cell assays. A methylcellulose erythroid colony system was used (13) for assays of marrow CFU-E and blood BFU-E. Co-cultures were performed as follows: 1×10^5 patient or control marrow T cell or Tγ cells were mixed with 1×10^5 patient or control marrow TD-LDMNC and scored for day 7 CFU-E. For BFU-E, 2×10^5 patient or control blood T cells were mixed with 2×10^5 patient or control blood null cells (6) and scored for day 14 BFU-E. Erythroid colonies were always absent in control cultures of T cells or Tγ cells plated alone. Each co-culture was plated in triplicate. Human urinary erythropoietin kindly provided by the Erythropoietin Subcommittee, (National Heart, Lung, and Blood Institute, Bethesda, MD) was present in cultures at a final concentration of 1 IU for marrow CFU-E and 2 IU for blood BFU-E. Aggregates containing eight or more benzidine-positive cells on day 7 were defined as 1 CFU-E. Aggregates containing >50 benzidine-positive cells on day 14 were defined as 1 BFU-E.

Diffusion chamber studies. In some studies, the in vivo mouse diffusion chamber (DC) culture system was used (14) to study the cellular interaction of patient B's T cells on granulocyte production. In this system, mature and immature granulocytes retrieved for the chambers after 14 d are identified on Wright-Giemsa-stained smear. In these studies, 4×10^5 patient blood T cells were inoculated into quadruplicate DC with 4×10^5 control T cells and 1×10^5 control TD-LDMNC. DC inoculated with 4×10^5 normal T cells plus 10^5 TD-LDMNC served as controls. Granulocytes did not proliferate in DC inoculated with only patient or control T cells.

Surface marker analyses. Surface immunoglobulin typing was performed on blood WMNC obtained by FH density gradient centrifugation by direct immunofluorescence using fluorescein-conjugated goat $F(ab')_2$ fragments (Meloy Laboratories, Inc., Springfield, VA) directed toward the human heavy or light chains (15). Blood or marrow E-rosetted cells or Tγ cells were further subtyped into helper (OKT4+) or suppressor/cytotoxic (OKT8+) monoclonal antibody-defined subsets (Ortho Pharmaceutical, Raritan, NJ) using an indirect immunofluorescence method with fluorescein-conjugated goat anti-mouse IgG (12, 16).

Serum inhibitor studies. Serum samples obtained from patients during active phase of PRCA and two type AB control donors were stored at −20°C before testing. IgG fractions were isolated by ammonium sulfate precipitation followed by DEAE cellulose column chromatography (17). The IgG fractions gave identical single bands of reactivity with anti-human whole sera and anti-human IgG on immunodiffusion plates (Hyland Diagnostics Div., Costa Mesa, CA). Patient or control IgG fractions were dialyzed against α-MEM and added to the cultures at a final concentration equivalent to 10% of patient's serum IgG level. Whole sera or IgG fractions were tested in the presence of a 1:10 dilution of rabbit complement (Low Tox, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada). In some serum studies, exogenous erythropoietin was omitted in order to test for the presence of erythropoietin in the samples. Serum or IgG fractions were continuously present throughout the culture period. 2×10^5 normal or patient marrow LDMNC obtained after initial treatment (but before complete recovery) were used as target cells.

Statistics. Comparison of cohorts was made using Student's *t* test.

RESULTS

Marrow CFU-E before and after resolution of PRCA. CFU-E in LDMNC from CLL-PRCA patients were barely detectable (3±1 CFU-E/10^5 LDMNC) during the active phase of PRCA (i.e., at presentation) compared with 12 normal controls (153±41/10^5 LDMNC, Fig. 1). 4 and 6 wk after initial treatment but before development of a reticulocytosis, CFU-E numbers were 48±3 and 28±7 CFU-E/10^5 LDMNC in patient A and B. After remission of PRCA, CFU-E rose to 90±10 and 42±7/10^5 LDMNC in patients A and B, respectively. These numbers, however, were still reduced compared with normal ($P < 0.05$).

Serum inhibitor studies. The effects of patient serum or IgG fractions on autologous erythroid colony formation from initial posttreatment patient marrows are summarized in Table I. Compared with normal control sera or IgG fractions, CLL-PRCA whole sera or IgG fractions obtained during the active phase of PRCA did not suppress erythroid colony growth from autologous marrow target cells ($P > 0.1$). Similar re-

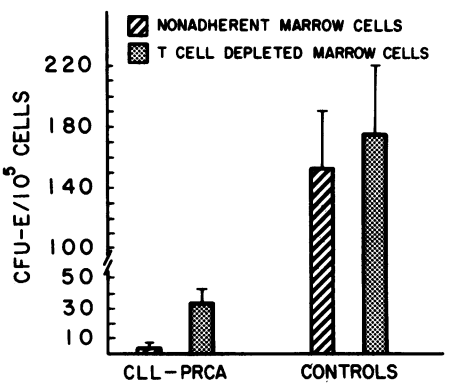

FIGURE 1 The effects of removal of marrow T cells by E-rosetting on growth of CFU-E in T-cell-depleted fractions. Compared with nonadherent cells that contained T cells, CFU-E numbers were increased 10-fold in CLL-PRCA patients but not in 12 normal controls.

TABLE I
Effects of Whole Sera or IgG Fractions from CLL-PRCA Patients on Erythroid Colony Growth from Autologous Marrows

Serum additions*	CFU-E/10^5 cells‡ Patient A marrow	Patient B marrow
Complement only	48±3	28±7
Patient serum	67±11 (139)	52±11 (185)
Normal serum	61±12 (127)	48±10 (171)
Patient IgG	45±9 (94)	22±7 (78)
Normal IgG	41±7 (85)	29±8 (103)
Patient serum (no EPO)	65±12 (135)	45±5 (161)

* Cultures were incubated with 10% patient serum (active phase of PRCA) normal AB serum, or their derivative IgG fractions. All samples were cultured with 0.1 ml of rabbit complement (C′) and 1.0 IU/ml erythropoietin (EPO). Each patient serum was also added to cultures in the absence of exogenous EPO (no EPO) to measure serum EPO activity. Nonadherent patient marrow cells retrieved after initial treatment but before complete recovery of PRCA served as targets.

‡ Values are means±1 SD of triplicate cultures; values in parentheses are percentages of C′ control. Colony growth with patient sera or IgG fractions did not differ significantly from normal controls.

sults were observed when normal marrows served as target cells (data not shown). The CLL-PRCA sera stimulated growth of autologous (Table I) or normal marrow CFU-E in the absence of exogenous erythropoietin suggesting that erythropoietin was present and antierythropoietin antibodies were absent.

T cell and Tγ cell composition of marrow and blood. A striking increase in E-rosetted (T cells) was observed in mononuclear marrow cells from both CLL-PRCA patients during the active phase of PRCA (Table II, columns 1 and 3). In contrast, in five untreated patients with Rai stage 0-III (3 stage III, 1 stage II and 1 stage 0 patients) B-cell CLL marrow T cells comprised only 7±2% of mononuclear cells. 90 and 35% of marrow T cells in the CLL-PRCA patients bore Fc receptors for IgG i.e., were Tγ cells. 53 and 55% of these Tγ cells reacted with the OKT8 suppressor antibody, whereas only 37 and 34% reacted with the OKT4 helper antibody. In the three patients with untreated B-cell CLL (Rai stage III) 51, 35, and 39% of marrow T cells were Tγ cells. Thus, although Tγ cells were increased proportionally in both CLL-PRCA and advanced stage III common B cell CLL marrows, the total numbers of Tγ cells were less in patients without PRCA. In patient A, after resolution of PRCA, the proportion of marrow T cells and Tγ cells decreased towards normal but were still increased (Table II, column 2). In patient B, the numbers of T cells and Tγ cells were normal after resolution of PRCA (Table II, column 4).

Before treatment, blood E-rosettes in the CLL-PRCA patients were decreased consistent with B-cell CLL (Table II, columns 1 and 3). However, the proportions of Tγ cells were markedly increased (Table II, columns 1 and 3). OKT4/OKT8 helper suppressor ratios were 0.78:1 and 0.86:1 in patients A and B, respectively, compared with controls (1.8:1). After resolution of PRCA posttreatment, the proportion of Tγ cells in blood decreased (Table II, columns 2 and 4). The proportion of T cells in blood had risen slightly to 21 and 11% (Table II, columns 2 and 4) but were still abnormally low.

TABLE II
T-Cell Surface Markers in B-Cell CLL

Test	Percentage of cells rosetting* Patient A PRCA(+)	Patient A PRCA(−)	Patient B PRCA(+)	Patient B PRCA(−)	Normal range‡
BM ER^+	70	22	25	7	5–15
BM $T\gamma^+$	90	40	35	20	10–30
PB ER^+	10	21	8	11	45–75
PB $T\gamma^+$	75	35	66	11	10–20

* Values represent the percentage of E-rosette-positive (ER^+) T cells from whole mononuclear cells or percent T cells rosetting with IgG-coated ox erythrocytes (Tγ cells) from bone marrow (BM) or blood (PB) before ($PRCA^+$) or after ($PRCA^-$) resolution of PRCA.

‡ Indicates range for 12 controls.

T-cell depletion studies. Removal of T cells from CLL-PRCA marrows before erythroid colony-forming assays, increased CFU-E numbers ~10-fold compared with CFU-E numbers in LDMNC (Fig. 1). The percent recovery of TD-LDMNC in patient A and B was 75 and 92%, respectively. Thus, concentration of CFU-E due to loss of TD-LDMNC could not explain this increase. Furthermore, based on the percentage of T cells in marrows of this patient only a 3.3- and 1.3-fold increase in patients A and B, respectively, would be expected due to enrichment of CFU-E alone. Moreover, separate studies in 12 normal volunteers did not show a similar augmentation of CFU-E numbers after removal of T cells ($P > 0.05$, Fig. 1).

Co-culture studies. To confirm that increased colony formation was due to removal of T suppressor cells, 10^5 T cells from CLL-PRCA marrows were retrieved and mixed with autologous marrow TD-LDMNC. The ratio of T cells of TD-LDMNC was 1:1 (Table III). Readdition of autologous CLL-PRCA marrow T cells, obtained during the active phase of PRCA, suppressed CFU-E proliferation to 56 and 67% of control cultures without T cells ($P < 0.05$). By contrast, an equal number of normal T cells stimulated CFU-E three- to sixfold. When CLL-PRCA Tγ cells were mixed with autologous marrow target cells, CFU-E growth was suppressed to 23 and 28% of controls ($P < 0.025$). Suppression of CFU-E from CLL-PRCA marrow cells by normal Tγ cells was not observed.

When CLL-PRCA T cells or Tγ cells were co-cultured with normal marrow TD-LDMNC in 1:1 ratios, CFU-E growth was suppressed to 34 and 47% of controls ($P < 0.025$, Table IV). Addition of normal T cells or Tγ cells to normal autologous marrows resulted in no significant change in CFU-E numbers.

Co-culture studies were repeated in patient A and B after they had achieved drug-induced remissions of their PRCA and when the proportions of Tγ cells in blood and marrow T cells were normal or nearly normal (Table II). At this time, CFU-E numbers in marrow TD-LDMNC (Table V) were increased 1.7- to 2.3-fold compared with CFU-E numbers in TD-LDMNC during PRCA. However, they were still significantly reduced compared with normals ($P < 0.05$). During remission of their PRCA, the effects of patients' T cells on CFU-E growth from autologous TD-LDMNC did not differ significantly from the effects of control T cells ($P > 0.05$, Table V). Similar results were observed when normal TD-LDMNC were used as target cells in co-cultures (data not shown).

Additional co-culture studies were performed in patient B to determine whether the T-suppressor effect was specific for CFU-E. As shown in Table VI, addition of blood T cells from patient B at the time of PRCA stimulated autologous or allogeneic BFU-E growth

TABLE III
Effects of T Cells or Tγ Cells from CLL-PRCA Patient Marrows on Erythroid Colony Growth from Autologous Marrows

Cell additions*	CFU-E/10^5 cells‡	
	Patient A bone marrow	Patient B bone marrow
None (control)	39±5	25±3
Patient T cells	26±5 (67)	14±2 (56)
Normal T cells	127±19 (325)	137±32 (548)
Patient Tγ cells	9±1 (23)	7±1 (28)
Normal Tγ cells	26±9 (67)	30±7 (120)

* 10^5 T cells or Tγ cells from patients or normal marrows were co-cultured with 10^5 T-cell-depleted marrow cells from patients A and B. Studies were performed with patients during the active phase of PRCA. Controls represent the numbers of CFU-E in T-cell-depleted marrows alone. CFU-E did not proliferate in T cells or Tγ cells cultured alone.

‡ Values are means±1 SD of triplicate cultures; values in parentheses indicate the percentage of control CFU-E. Addition of active phase patient T cells or Tγ cells decreased CFU-E numbers from patient marrows as compared with addition of normal T cells or Tγ cells ($P < 0.025$).

TABLE IV
Effects of T Cells or Tγ Cells from CLL-PRCA Patients on Erythroid Colony Growth from Normal Marrows

Cell additions*	CFU-E/10^5 cells‡	
	Normal bone marrow I	Normal bone marrow II
None (control)	152±31	208±20
Patient T cells	51±11 (34)	97±15 (47)
Normal T cells	139±22 (91)	209±35 (100)
Patient Tγ cells	24±5 (16)	45±15 (22)
Normal Tγ cells	155±21 (102)	221±20 (106)

* 10^5 T cells or Tγ cells from patients or normal marrows were co-cultured with 10^5 T-cell-depleted marrow cells from normal donor I or II. Studies were performed with patients during the active phase of PRCA. Controls represent the numbers of CFU-E in normal T-cell-depleted marrows alone. CFU-E did not proliferate in T cells or Tγ cells cultured alone.

‡ Values are means±1 SD of triplicate cultures; values in parentheses indicate the percentage of control CFU-E. Addition of active phase patient T cells or Tγ cells decreased CFU-E numbers from normal marrows as compared with addition of autologous normal T cells or Tγ cells ($P < 0.025$).

TABLE V
Effects of T Cells from CLL-PRCA Patients on Erythroid Colony Growth from Autologous Marrows after Resolution of PRCA

Cell additions*	CFU-E/10^5 cells‡	
	Patient A bone marrow	Patient B bone marrow
None (control)	90±10	42±7
Patient T cells	107±15 (118)	47±10 (112)
Normal T cells	130±10 (144)	55±14 (154)

* 10^5 T cells from patients or normal marrows were co-cultured with 10^5 T-cells-depleted marrow cells from patients A or B. Studies were performed with patients after resolution of PRCA. Controls represent the numbers of CFU-E in T-cell-depleted marrows alone. CFU-E did not proliferate in T cells cultured alone.
‡ Values are means±1 SD of triplicate cultures; values in parentheses indicate the percentage of control CFU-E. The effects of patients T cells obtained after resolution of PRCA on erythroid colony growth did not differ significantly from controls ($P > 0.05$).

from blood null cells, although the stimulatory effect was less than that observed in the normal T cells ($P < 0.05$) under identical conditions. Furthermore, when 4×10^5 T cells from patient B were mixed with equal numbers of normal T cells before inoculation into DC and scored for total granulocytes (mature and immature), no significant differences in granulocyte precursor cells (myeloblasts-polymorphonuclear leukocytes) were observed from 10^5 TD-LDMNC of two normal donors compared with DC containing only 4×10^5 normal T cells (Table VII).

TABLE VI
Reduced T Cell Burst-promoting Effect of CLL-PRCA T Cells

Additions to null cells*	Controls	Patient B
None	60±24	10±2
Patient T cells	110±21	70±24
Control T cells	278±52	185±25

* 2×10^5 normal control T cells or patient T cells retrieved during active phase of PRCA were co-cultured with 2×10^5 normal or patient blood null cells and scored for day 14 BFU-E. BFU-E were absent in T cells cultured alone.
‡ Values indicate mean±1 SD. BFU-E/2×10^5 null cells in triplicate plates. Patient's T cells stimulated normal or control T cells but the burst-promoting effect was significately less than observed with normal T cells under identical conditions ($P < 0.05$).

TABLE VII
Lack of Suppression by CLL-PRCA T Cells on Normal Granulocyte Proliferation

Additions to control TD-LDMNC*	Control I	Control II
None	5±1	23±5
Control T	12±3	33±6
Control T + patient T	9±2	36±6

* 10^5 blood TD-LDMNC from control I or II were inoculated into DC with 4×10^5 control T cells alone or with 4×10^5 patient T cells during active phase of PRCA. Granulocyte precursors did not proliferate in control or patient T cells inoculated into DC alone.
‡ Values indicate mean±SD. Granulocyte precursors/10^5 TD-LDMNC for four DC per culture. Patient T cells did not suppress normal granulopoiesis under these conditions.

DISCUSSION

The results of in vitro culture studies and T-cell subset analyses in two patients with B-cell CLL who developed PRCA implicate Tγ cells as mediators of the suppressed erythropoiesis. At the time of PRCA both patients' Tγ cells were present at abnormally high concentrations in marrow and blood. Furthermore, depletion of T cells from marrow suspensions significantly augmented in vitro erythroid colony growth in the patients but not in normal controls. This augmentation did not appear to be due to enrichment of erythroid progenitor cells. Co-culture studies showed that addition of either unseparated CLL-PRCA T cells or Tγ cells to autologous or normal T-cell-depleted marrows suppressed CFU-E proliferation; similar studies in normals did not result in a comparable decrease in CFU-E numbers. Moreover, after resolution of PRCA, T-cell subset analyses of marrow and blood indicated that Tγ cells were reduced substantially. In both patients after drug-induced resolution of the PRCA, T-cell suppressor activity to CFU-E was not detectable when co-culture studies were repeated.

Serum inhibitors to erythroid progenitor cells were not found. Previous studies in patients with idiopathic PRCA have implicated autoantibodies directed against marrow pronormoblasts or erythroid progenitor cells (18–20) but these have not been reported in CLL-PRCA (4–6). In other patients who develop autoimmune hemolytic anemia, it is possible that antigens on CFU-E are targets for antibodies (21). In our cases, direct Coombs tests were persistently negative and evidence for antibody-mediated suppression of erythropoiesis was lacking. In fact, sera from CLL-PRCA patients taken during the active phase of PRCA stimulated erythroid colony formation in the absence of

exogenous erythropoietin. These studies indicate that erythropoietin was present and antierythropoietin antibodies were unlikely (20, 22).

Previous investigators have emphasized the need for cautious interpretation of in vitro co-culture studies due to the possible effects of allosensitization and histoincompatibility (23, 24). Although the allosensitization of T suppressor cells by transfusion therapy cannot be excluded in patient A, studies in patient B were performed before transfusions. Furthermore, the in vitro inhibitory effects of T cells were found using both autologous and allogeneic conditions; thus, it is unlikely that suppression can be attributed to histocompatibility differences alone.

The B cell origin of the lymphocytic leukemia in our patients was confirmed by the findings of a monoclonal immunoglobulin (IgM lambda) on the surface of a majority of blood lymphocytes, and the absence of typical clinical and morphologic features that may be seen in T-cell CLL. Thus, these cases differ from those previously described by Hoffman et al. (8) and Nagasawa et al. (9), who found CFU-E suppression to be mediated by T cells from two patients with T-cell CLL. In the former case report, B cells from patients with B-cell CLL did not suppress CFU-E proliferation in vitro (8). We have also been unable to demonstrate suppression of erythropoiesis in vitro by B cells from normals (11) or patients with B-cell CLL (unpublished observations).

Our E-rosette T-cell depletion studies performed on normal controls (Fig. 1) are consistent with the concept that the mature erythroid stem cells (CFU-E) can proliferate in the absence of T cells (25). CFU-E numbers were, in fact, increased after T cell depletion in normals, which may reflect some enrichment of CFU-E by the separation procedure. However, the 10-fold increase in CFU-E numbers of CLL PRCA patients after T cell depletion could not be attributed to either enrichment by loss of T cells or TD-LDMNC in the separation procedure. Moreover, readdition of normal T cells to TD-LDMNC marrow cells consistently increased CFU-E numbers from normal or patient marrow cells (Tables III and IV). Therefore, suppression of CFU-E growth under these conditions is quite unexpected. The T-cell enhancing effect of CFU-E is not due to addition of CFU-E, since control T cells cultured separately were devoid of erythroid colonies. It is possible that T cells may enhance the responsiveness of CFU-E to erythropoietin or recruit resting pre-CFU-E.

The localization of the CFU-E T-suppressor activity in CLL-PRCA to the Tγ cell subset is consistent with the observed effects of these cells in other cell cooperative systems. Tγ cells have been shown previously to suppress B-lymphocyte differentiation and immunoglobulin production (26), inhibit granulocyte progenitor cells (CFU-C) proliferation in patients with aplastic anemia (27), and reduce BFU-E proliferation in normals and patients with CLL (10, 12). The inhibitory effect of Tγ cells may be mediated by the OKT8+ reactive suppressor cells located in these fractions (12). In this regard, it is noteworthy that immunoglobulin production was also reduced when OKT8+ Tγ cells were increased in blood and marrow CLL-PRCA T cell pools. Since OKTM1+ natural killer (NK) cells may also be located in the Tγ cell fractions (12, 28) the possibility that the suppressor effects were mediated by NK cells is not excluded.

T cells and Tγ cells were increased in bone marrows of the B-cell CLL-PRCA patients. Since the percentage of T cells in peripheral blood was reduced compared with marrow, it is unlikely that these findings reflect the rare occurrence of a leukemic B-cell clone displaying an E-rosette receptor (29). Rather, it seems more likely that the increase in marrow T cells reflects an abnormal distribution or migration into the marrow. In patients with untreated B cell CLL, we also found an increased proportion of Tγ cells. However, the total numbers of marrow T cells were not increased in these patients, as was observed in the CLL-PRCA patients. These findings may explain why most patients with B-cell CLL did not develop PRCA even though the proportions of Tγ cells in peripheral blood and marrow are increased (30–32). On the other hand, most, if not all patients with B-cell CLL will eventually develop a hypoproliferative anemia. The variable hypoproliferative anemias found in these patients may reflect the gradual accumulation of suppressor Tγ cells in the marrow.

The cause for the expansion of Tγ cells in the marrows of CLL-PRCA patients is not clear. Recent evidence that T-cell antigens defined by monoclonal antibodies are present on B cells of some CLL patients (33) raises the possibility that the expansion of T cells in B-cell CLL (30) and the abnormal T-cell function (34) may be a result of the underlying leukemic process. Alternatively, the T cell and Tγ cell expansion may reflect an unregulated secondary response in the expanded malignant B-cell clone.

Whatever the reason for T-cell expansion, our studies suggest that suppression of erythropoiesis may be primarily restricted to mature erythroid stem cells since (*a*) granulocytic and megakaryocytic maturation were normal on marrow smears and there was no clinical neutropenia or thrombocytopenia; (*b*) no in vitro evidence for suppression of granulopoiesis was ob-

served in DC studies under allogeneic conditions; and (*c*) primitive BFU-E proliferation was suboptimal but not suppressed in the presence of CLL PRCA T cells. Differences in target antigens on erythroid and granulocyte stem cells may account for these findings (35).

ACKNOWLEDGMENTS

We thank Lesley Bieler and Sherri Matis for expert technical assistance and Mary T. Almade for preparation of the manuscript.

This work was supported, in part, by grant S07-RR05394 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, the Veterans Administration, and by grant Y-34 Health Research and Services and Foundation, United Way, Pittsburgh, PA 15219.

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