T lymphocytes were derived by E rosetting from the peripheral blood (PB) and bone marrow (BM) of 15 patients with chronic granulocytic leukemia (CGL) in the chronic phase of their disease. T cells were also obtained from 12 healthy individuals. T cells were incubated overnight either in culture medium (RPMI) or RPMI plus pokeweed mitogen (PWM). The supernatants were then recovered and the cells washed in fresh RPMI. T cells from normal donors and from CGL patients were then cocultured with normal allogeneic marrow cells grown in soft agar for CFU-C colony formation. Target marrow cells were also grown in agar in the presence of T-derived supernatants. The results of this study can be summarized as follows. (1) Normal PB and BM T cells efficiently suppressed autologous and allogeneic CFU-C growth after PWM stimulation. (2) T cells derived from peripheral blood or marrow of CGL patients failed to inhibit CFU-C growth, whether pretreated with PWM or not. (3) The supernatants of PWM-treated normal T cells strongly inhibited CFU-C colony formation, whereas the supernatants of PWM-treated CGL T cells had no CFU-C/suppressor activity. These data indicate that T cells from CGL patients cannot be primed to become CFU-C suppressor cells after PWM stimulation in vitro and cannot release a soluble inhibitor of granulopoiesis produced by PWM-primed normal T cells.

There is accumulating evidence for a regulatory role of T cells in hemopoiesis. Enhancing as well as suppressor activity have been described both in vitro and in vivo both in animals and in man. Similar to other functions of T cells, enhancement and suppression of hemapoiesis may be produced by different T-cell subsets with distinct surface markers. Along this line we have recently reported that the suppressor activity on CFU-C is restricted to T cells with Fc receptors for IgG (Tc cells) and is mediated via a soluble factor released in culture.

An excess of CFU-C/suppressor activity is found in patients with immune severe aplastic anemia (SAA) and is therefore thought to be associated with marrow aplasia and pancytopenia. It is possible that the opposite abnormality, that is, lack of CFU-C suppressor T cells, may be associated with uncontrolled expansion of hemopoietic cells.

To test this hypothesis, T cells from chronic granulocytic leukemia (CGL) patients and from normal individuals were activated in vitro with pokeweed mitogen (PWM) and then added to marrow cultures to assess their CFU-C suppressor activity. Unlike normal T cells, which showed potent CFU-C inhibition after mitogen priming, T cells from CGL patients consistently failed to exhibit any significant CFU-C suppression, whether derived from the peripheral blood or marrow and whether pretreated with PWM or not.

**Materials and Methods**

**Patients**

Fifteen patients with chronic granulocytic leukemia (CGL) were admitted to this study. The Ph' chromosome was detectable in 12/15 patients. Ages ranged from 12 to 52 yr. Eight patients were males, 7 females. White blood cell counts ranged from $6 \times 10^9$/liter to $150 \times 10^9$/liter. Seven patients were in the chronic phase of their disease, 4 off maintenance therapy, and 3 on busulfan, 2 mg p.o./day. Four patients were studied at presentation of CGL.

**Normal Controls**

Twelve healthy individuals served as normal controls. Both marrow and peripheral blood cells were obtained and processed as described below.

**Target Cells**

Marrow cells were obtained from the posterior crests in heparinized syringes. The cells were run on a Ficoll-Hypaque (800 g) gradient, and the light density cells recovered and washed 3 times in McCoy's medium plus 5% fetal calf serum (FCS). The cells were then incubated in plastic Petri dishes at 37°C for 30 min in 5% CO2 at a concentration of $3 \times 10^6$ cells/ml in McCoy's medium plus 20% FCS to remove adherent cells. Nonadherent cells were washed out with the supernatant and served as target cells for coculture experiments.

**Effector Cells**

Peripheral blood and bone marrow T lymphocytes served as effector cells. Light density peripheral blood (PB) and marrow (BM) cells obtained from a Ficoll-Hypaque (800 g) gradient were depleted of adherent cells as described. Thereafter, the cells were incubated with neuraminidase-treated sheep erythrocytes. E-rosetting cells were separated from nonrosetting cells on a Ficoll-Hypaque gradient and freed of indicator sheep erythrocytes by lysis with ammonium chloride buffer. Thereafter, E-rosetting cells were
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Mitogen Priming

Purified PB and BM T cells were then incubated overnight in RPMI plus 20% FCS or in RPMI plus 20% FCS plus pokeweed mitogen (PWM) 20 μg/ml (10⁶ cells in 0.1 ml of medium in round-bottomed Terasaki wells) at 37°C in 5% CO₂. The next morning the supernatants were recovered and the cells washed in fresh medium. RPMI-primed and PWM-primed T cells and their supernatants were then added to marrow cultures grown in agar.

Bone Marrow Cultures

BM cells from normal controls were cultured in agar (10⁶ cells/35 mm Corning plates) according to Pike and Robinson¹⁷ with leukocyte feeder layers, or in human placenta conditioned medium (HPCM) as a source of colony-stimulating factor (CSF). HPCM was prepared according to the technique of Burgess et al.¹⁸ Colonies of over 40 cells were scored on day 10 of culture.

Cocultures

These were set up with 10⁶ target and 10⁴ effector cells mixed together and plated in semisolid agar soon thereafter. The following combinations were thus studied: RPMI- or PWM-primed normal BM or PB T cells plus normal marrow cells; RPMI- or PWM-primed T cells from CGL (both PB and BM) plus normal marrow cells. The supernatants of PWM-primed normal or CGL T cells were also added to normal marrow cells (0.1 ml of supernatant obtained from an overnight culture of 10⁶ T cells/10⁶ normal marrow cells) immediately before these were plated in agar. Admixtures of 5 × 10⁶ normal cells and 5 × 10⁶ CGL cells (normal E⁺ cells plus CGL E⁺ cells; normal E⁻ cells plus CGL E⁻ cells; CGL E⁺ cells plus normal E⁻ cells) were also primed with PWM in overnight cultures and then tested for CFU-C suppressor activity. Statistical evaluation of the results was performed with a Student’s t test. Suppression in cocultures was expressed as (observed growth/expected growth) × 100.

Light Microscopic and Cytochemical Analysis

T cells obtained from normal controls and CGL patients were sedimented onto glass slides using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, Pa.). The slides were then stained with May-Grünewald-Giemsa for morphological studies. Alfa-naphthyl acid esterase (ANAEE) and acid phosphatase (AP) activity were demonstrated as previously described.¹⁶

Rosetting With IgG-Ox Erythrocytes

Normal and CGL T cells were rosetted with ox erythrocytes coated with anti-ox rabbit IgG as previously described.¹⁷ The cells were then placed between slide and coverslip and 200 cells/slide screened for EA-IgG rosettes.

Controls

T cells from normal donors and CGL patients were grown in semisolid agar in the presence of CSF to exclude contamination with colony-forming cells.

RESULTS

T-Cell Preparations

Isolated T cells from normal controls and CGL patients were again rosetted with neuraminidase-treated sheep erythrocytes; in normal controls more than 90% of the cells formed E rosettes. In CGL patients, one rosetting procedure yielded 75% of T cells, and double E-rosetting allowed further purification up to 95% of T cells. In addition, the proportion of T₉ cells was assessed both by cytochemical and immunologic markers. T cells obtained from normal marrow or peripheral blood contained, respectively, 35% ± 8% and 12% ± 7% of cells with the Fc receptor for IgG. T cells derived from the marrow or peripheral blood of CGL patients contained 30% ± 12% and 10% ± 4% of T₉ cells. These results were confirmed with cytochemical investigations.

Colony Formation

The number of CFU-C colonies from normal marrow on day 10 was 69 ± 18 in cultures grown with leukocyte feeder layers and 62 ± 12 in cultures supplemented with HPCM. T cells from CGL and normal donors produced no colonies when grown in agar. T-cell supernatants had no colony-stimulating activity and failed to sustain the growth of normal marrow cells in the absence of HPCM.

Effect of Normal T Cells on CFU-C

Cocultures of normal T cells primed with RPMI with normal marrow cells failed to produce any effect on colony formation. PWM-treated normal T cells reduced CFU-C growth to 23% ± 14% (range 0%–52%) of expected growth (EG ± SD) when T cells were derived from the peripheral blood, and to 31% ± 18% (range 0%–47%) of EG when derived from the marrow. The supernatant of PB RPMI-primed T cells showed no suppression on colony formation. The supernatant of PWM-treated PB and BM T cells reduced colony formation to 31% ± 21% (range 0%–51%) of EG and 21% ± 18% (range 0%–47%) of EG, respectively (Figs. 1 and 2). There was no statistical difference between colony formation of marrow cells and that of marrow cells plus CGL T cells (p > 0.7) or CGL T supernatant (p > 0.6). Although some degree of inhibition could be detected in two patients at presentation, this was not statistically greater than...
suppression of T cells from patients in the chronic phase of the disease ($p > 0.05$), but still significantly different from suppression of normal T cells ($p < 0.001$).

**Influence of CGL Cells on Normal Cells**

In order to exclude that non-T cells contaminating the $E^+$ cell fraction from CGL patients were responsible for active abrogation of the CFU-C suppressor effect, $E^+$ and $E^-$ cells from CGL patients were mixed with $E^+$ cells from normal donors: after an overnight incubation with PWM, the cells were washed and added to normal marrow cells.

Colony formation was reduced to $17\% \pm 6\%$ EG, $23\% \pm 11\%$ EG, and $10\% \pm 10\%$ EG (mean $\pm$ SD of 3 experiments), respectively, by $E^+$ normal cells alone, by $E^+$ normal cells mixed with $E^-$ CGL cells, and by $E^+$ normal cells mixed with $E^-$ CGL cells.

**Influence of Normal Cells on CGL Cells**

The possibility that CGL T cells failed to release the inhibitor of CFU-C (Td/CIA) because of a defect of accessory helper cells was also taken into consideration. Normal $E^+$ cells were thus added to CGL $E^-$ cells in the attempt of facilitating suppressor T-cell priming induced by PWM. Colony formation was reduced to $77\% \pm 11\%$ EG and $91\% \pm 11\%$ EG, respectively, when CGL $E^-$ cells alone or CGL $E^-$ cells mixed with normal $E^+$ cells were added to normal marrow.

**Fig. 2.** Effect of supernatants of mitogen-treated T cells on normal bone marrow. The supernatant of CGL T cells show no suppressor activity ($p > 0.8$) as compared to definite inhibition of supernatants from normal T cells ($p < 0.001$). The difference between the effect of CGL and normal T-cell supernatants on CFU-C is significant at the 0.001 level. Abbreviations as in Fig. 1. First column from the top: $21\% \pm 18\%$ EG. Second column: $95\% \pm 38\%$ EG. Third column: $31\% \pm 21\%$ EG. Fourth column: $85\% \pm 34\%$ EG.
DISCUSSION

The existence of T cells capable of suppressing the in vitro growth of myeloid and erythroid progenitor cells has been described in patients with severe aplastic anemia. In some of these, an in vivo activity of suppressor T cells on pluripotent stem cells has been postulated on the basis of complete autologous hematologic reconstitutions following immunosuppressive therapy. It is certainly of interest, and perhaps more than a casual association, that SAA patients responding to immunosuppression have detectable CFU-C suppressor T cells releasing a potent soluble inhibitor, which we shall refer to as T-derived colony inhibitory activity (Td/CIA). T cells derived from normal individuals can also release Td/CIA, but only after mitogen stimulation, whether derived from the marrow or peripheral blood. Although in vitro tests do not necessarily reflect physiologic events, these experiments indicate that the CFU-C inhibitor activity belongs to the repertoire of normal T cells and that an excess of suppressor activity may be associated with immune-mediated marrow failure.

In the present report we have shown that T cells from CGL patients, with a few exceptions, have lost this activity. There may be several explanations for this finding: lack of a distinct suppressor T-cell subset; defective helper activity of non-T cells; existence of cells inhibiting the production of Td/CIA; and finally, a functional abnormality of CGL T cells.

We do not believe CGL patients lack T cells with Fc receptors for IgG (T) cells known to release Td/CIA. Indeed, the percentage of T cells in the marrow and peripheral blood of CGL patients was quite comparable to that of normal controls.

As to the second and third hypothesis, the addition of normal non-T cells to CGL E' cells did not result in the generation of suppressor T cells; conversely Td/CIA release by normal T cells could not be rescued by the addition of CGL E' or CGL E' cells.

It should be noted that we have not yet investigated thoroughly the role of non-T-cell help in the production of Td/CIA, for example with T cells deprived of monocytes by means of monoclonal antibodies, nor have we any information on the HLA-DR restriction of possible T/non-T cell interactions. Thus, random pairing of CGL patients and normal donors may have been responsible for failure of normal and CGL cell subpopulations to cooperate. Therefore, although one cannot altogether exclude an abnormality of CGL non-T-cells resulting in a lack of Td/CIA production, the evidence presented in this study would be in favor of the last possibility, that is, a functional defect of CGL T cells. The expression of such a defect among patients would appear to be heterogeneous, as indicated by two cases in this series releasing detectable levels of suppressor factor. Isoenzymes and chromosome marker studies indicate that CGL is a clonal disease involving a stem cell from which granulocytes, monocytes, erythrocytes, and megakaryocytes arise. The Ph' positive cell also appears to give rise to lymphocytes, as indicated by the frequent lymphoid blastic transformation of the disease and by the cases presenting as Ph'-positive acute lymphoblastic leukemia (ALL). Surface marker analysis of such blasts has revealed similarities with common ALL, including a high content in the enzyme terminal deoxynucleotidyl transferase (TdT), suggested to be a marker for immature T cells. A pre-B-cell line has been recently established from a patient in blast crisis of CGL, and a Ph'-positive T-ALL has also been reported.

However, blood lymphocytes from CGL patients stimulated with PHA have only rarely been reported to be Ph'-positive, and this has been interpreted as evidence against the origin of T cells from the Ph'-positive stem cell.

Recently, two subpopulations of T cells have been reported in patients with CGL: one composed of normal T lymphocytes, either long-lived or derived from a progenitor not involved in the disease, and the other one arising from the leukemic cell clone. Our finding of a major functional abnormality of T cells in CGL both at presentation and during the chronic phase of the disease lends further support to this view. Indeed, CGL-derived T lymphocytes can perform a number of normal in vitro tests, such as proliferation in response to alloantigens or mitogens and colony formation in agar. However, residual normal T-cell activities give no information on the origin of the cells performing the test, especially after the finding of Ph'-positive PHA blasts. On the contrary, failure of T lymphocytes to release Td/CIA is an indication of a definite abnormality. Although we cannot exclude that such an abnormality has been acquired by normal T cells as a consequence of the disease, it would seem reasonable to take these data as further suggestions for the inclusion of at least one subpopulation of T cells in the leukemic cell clone.

We have recently shown that Td/CIA derived from SAA T cells and from PWM-primed normal cultures fails to inhibit the growth of CGL myeloid progenitor cells. Thus, two distinct abnormalities can be detected in CGL within the granulopoiesis/T-cell system, at least in vitro: lack of responsiveness of CFU-C to inhibition and failure of T cells to release the inhibitor.

In conclusion, although the physiologic relevance of
Td/CIA is not established, our previous studies and the present report indicate that T cells spontaneously releasing Td/CIA in culture can be derived from patients with marrow failure and that T cells unable to produce the inhibitor are detected in patients with uncontrolled expansion of the stem cell pool. More work on patients with other myeloproliferative disorders may help to explain the latter association.

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Generation of CFU-C suppressor T cells in vitro. III. Failure of mitogen-primed T cells from patients with chronic granulocytic leukemia to inhibit the growth of normal CFU-C

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