Granulopoiesis in Chronic Myeloid Leukemia. II.
Serial Cloning of Blood and Bone Marrow Cells in Agar Culture

By Tor Olofsson and Inge Olsson

Colony-forming cells (CFC) and CFC in S-phase were assayed in chronic myeloid leukemia (CML). A correlation was found between leukocyte counts and CFC of blood, suggesting that the leukocytosis of CML depended on expansion of the committed granulopoietic stem cell compartment. Serial studies performed in four cases demonstrated a decrease of the CFC in S-phase during early stages of developing leukocytosis, which was consistent with the operation of growth control mechanisms. During later stages, serial studies revealed that sudden increments of CFC S-phase coincided with rapidly growing leukocytosis, which was consistent with leukemic cell populations escaping growth control. 3H-thymidine labeling indices for differentiated precursor cells showed slight variations not coinciding with variations of the CFC S-phase fraction. Cyclic oscillations of the white cell count were observed in one case. The white cell count and the fraction of CFC in S-phase displayed a direct relationship, indicating that the occurrence of cycles was not likely to be due to a negative feedback mechanism elaborated by mature granulocytes. In another case, marked cycling of the CFC S-phase fraction was found without distinct oscillations of the white cell count. The present work has emphasized the necessity for serial assays of parameters of cell kinetics in vitro in CML since changing relationships were found between stem cell and differentiated cell kinetics during different phases of the disease.

Chronic Myeloid Leukemia (CML) is characterized by a successive accumulation of granulopoietic cells with no apparent arrest in maturation or mature granulocyte function.1 The leukocytosis is due mainly to an increased rate of granulocyte production, but after significant expansion of the total blood granulocyte pool, prolonged survival of the cells appears to play an additional role.2 Granulocytic progenitor cells from patients with CML proliferate in agar cultures3 to produce colonies of granulocytes and macrophages. The number of colony-forming cells (CFC) of blood is 1000 60,000 times greater than normal,7 indicating an overproduction of CFC by the leukemic tissue. Other evidence suggests that intact granulocytes normally produce substances, which by feedback inhibition have a bearing on granulocyte production.8 11 Theoretically, depression of inhibitory factors, increase of stimulatory factors, and altered target-cell responsiveness could be involved in the overproduction of granulopoietic cells in CML. Mature granulocytes from both healthy and CML donors inhibit colony formation from marrow cells, sug-

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gesting that a negative feedback regulation could operate, but apparently it is not efficient enough to control cell production.

The aim of the present work was to study parameters of stem cell kinetics in vitro in CML to provide a basis for the evaluation of regulation of granulopoiesis in this disease. The CFC concentration and CFC cell cycle characteristics, as well as the 3H-thymidine labeling indices of differentiated progenitor cells have been determined. Serial studies on individual patients have been performed in order to correlate the results with the course of the disease.

**MATERIALS AND METHODS**

**Patient Selection**

Twelve CML patients were studied, four of them with serial studies. All demonstrated low leukocyte alkaline phosphatase scores, and cytogenetic analyses of marrow specimens showed the presence of the Ph' chromosome in all cases. Spleen size was determined by physical examination by one physician: spleen size was scored (+) if less than 10 cm, (++) if 10-15 cm and (+++) if more than 15 cm below the xiphoid process.

Bone marrow specimens for the determination of CFC concentration, and cell cycle characteristics were obtained from a control group of patients defined as consisting of subjects with morphologically normal marrows and normal neutrophil counts.

**Collection of Blood**

Fifty milliliters of heparinized blood (20 I.U. of heparin/ml without preservative) were collected from healthy donors, and various amounts of heparinized blood were obtained from the CML patients.

**Collection of Bone Marrow**

Marrow aspirated from the sternum was collected in a sterile tube containing 3 ml of McCoy's medium, 100 I.U. of heparin and 75 units of Varidase (Lederle Laboratories, Pearl River, N.Y.). The tube was left standing for 10 min to let the marrow fragments sediment. The supernatant was withdrawn, and the fragments were washed gently twice in McCoy's medium. The washed marrow particles were then disrupted by repeated suction through a needle. This procedure was used with CML marrow to minimize contamination with peripheral blood. Normal marrow aspirates were suspended immediately in the collecting medium.

**Cell Separation**

One part of heparinized blood was mixed with one part of 2% Dextran 250 (Pharmacia, Uppsala, Sweden) in 0.15 M sodium chloride and allowed to stand for 45-60 min at room temperature. The leukocyte-rich plasma was then separated using the Isopaque-Ficoll system of Böyum. Ten parts of 33.9% Isopaque solution (Nyegaard and Co., Oslo, Norway) with a density of 1.2 g/ml were mixed with 23 parts of 8% Ficoll (Pharmacia, Uppsala, Sweden) in water solution and the density adjusted to 1.077 g/ml. Thirty milliliters of normal or CML leukocyte-rich plasma (diluted with culture medium to a final leukocyte concentration of 10,000-20,000 cells per µl) were layered on top of 20 ml Isopaque-Ficoll mixture in 50-ml sterile plastic tubes. Marrow suspensions were separated in the same manner, omitting Dextran sedimentation; 7 ml of bone marrow suspended in culture medium were layered on top of 5 ml Isopaque-Ficoll solution in 16-ml plastic tubes. The tubes were centrifuged with a swing out rotor for 10 min at 70 g and for 15 min at 700 g. The cells collected on top of the Isopaque-Ficoll which consisted of lymphocytes, monocytes, and immature marrow cells were designated A cells. The cells collected at the bottom of the tube (mature granulocytes and contaminating erythrocytes) were designated B cells. The main reason for employing the cell separation technique was to sort out the B cells which inhibit colony formation. The A-cell fraction was removed, washed three times in culture medium and used for cloning in agar; A cells from normal peripheral blood were used for preparation of feeder layers. The colony-forming cells were quantitatively recovered (> 99%) in the A-cell fraction.
On the basis of WBC and differential counts of the A-cell fraction, colony formation from the A cells was expressed as CFC/μl blood according to the formula:

\[
\frac{\text{CFC}}{(\text{Mb} + \text{PMC} + \text{MC})/\text{culture dish}} = \frac{\text{colonies}/\mu l \text{ blood}}{(\text{Mb} + \text{PMC} + \text{MC})/\mu l \text{ blood}}
\]

(Mb, myeloblasts, PMC, promyelocytes, MC, myelocytes). Because of the abnormal cell density distribution of immature myeloid cells in CML, the recovery of these cells varied in the A-cell fraction. Based on detailed analysis of the separations performed, it was found that the calculations of colonies/μl blood were overestimated by 7.4% ± 1.6% SEM. The results given here have not been corrected for this minor error.

**Cell Culture Technique**

The culture technique and the modification of McCoy's SA medium as described by Pike and Robinson were utilized. The feeder layers contained 3 × 10^5 A cells obtained from the peripheral blood of the same donor throughout this study; 1 ml of cells in 0.5% agar (Difco Bacto Agar) in culture medium was plated into 35-mm Falcon plastic petri dishes. The over layers contained 1 × 10^5 A-cells of CML blood, CML marrow, or normal marrow. After gelation at room temperature the plates were incubated for 10 days at 37°C in a fully humidified atmosphere with 7.5% CO₂ in air. Colony counting was performed using a microscope and aggregates containing at least 40 cells were counted. The mean value of three to four dishes was used for each determination. Analysis of cluster and colony formation from CML blood and marrow showed that clusters (3-10 cells) reached the maximum on day 5, clusters (11-20 cells) on day 8, clusters (21-30 cells) and (31-40 cells) on day 8-9 and then declined. Colonies reached maximum size on day 8-9 and remained at a constant level at least until day 14. Identification of cellular constituents of individual colonies was performed after removal with a Pasteur pipette and staining by the addition of 2% orcein in 60% acetic acid.

For determination of the proportion of the progenitor cells in DNA synthesis the ∆H-thymidine suicide technique was used. One and a half milliliters of A cells in thymidine-free McCoy’s medium containing 3-5 × 10^6 cells was incubated in a 16-ml plastic tube with 75 μCi of ∆H-thymidine with a specific activity of 17 Ci/mole (Radiochemical Centre, Amersham, England). A control was run simultaneously without ∆H-thymidine. After 30 min of incubation at 37°C, the cells were...
washed twice in 15 ml McCoy's medium with 100 μg/ml of thymidine, once in culture medium without thymidine and then used for agar culture as described above. The cell suspensions were also used for determination of the 3H-thymidine (3H-TdR) labeling index (LI.) of myeloblasts, promyelocytes, and myelocytes by autoradiography using autoradiographic emulsion K2 (Ilford Ltd., Ilford, England). Cells with 4 grains were considered labeled. Myelocytes and promyelocytes were counted together.

In order to determine the methodologic variations of the 3H-thymidine suicide technique, a normal marrow was separated as described above and split in seven identical fractions. To six of the tubes 3H-thymidine was added (50 μCi/ml), and all tubes were incubated at 37°C for 30 min and the cells washed and plated as described. The SEM of the calculated S-phase per cent was ± 1.31. Control experiments showed that cold thymidine inhibited the suicidal effects of 3H-thymidine in a dose-dependent way.

RESULTS

The relationship between blood colony-forming ability and leukocyte count is shown in Fig. 1. The results are based on studies of 12 CML patients and included are the serial observations shown in Figs. 2-5. There is a correlation between CFC/μl blood and leukocyte counts (calculation based on the linear values, r = 0.62, p < 0.001). Inspection of Figs. 2-5 further emphasizes that an increase in blood CFC with time is generally followed by an increase in circulating leukocytes.

Four CML patients underwent serial studies of in vitro growth of peripheral blood CFC. In one patient, blood and marrow cells were studied concomitantly. The results are shown in Figs. 2-5 presented as CFC/μl blood and as per cent CFC in S-phase. Data for hemoglobin concentration, spleen size, WBC
Fig. 3. Serial studies on a 35-yr-old female (C.T.) with CML of 5-yr duration. Six months prior to the present study treatment with busulfan had been given for a period of 10 wk. Hemoglobin concentration (e—e), degree of splenomegaly, neutrophil count (e—e), total leukocyte count (e—e), and treatment given is indicated. The hydroxyurea dose was 4 g every third day. Data are also given for CFC per microliter of blood (e—e) and per cent CFC in S-phase (e—e). Chromosome analysis of marrow carried out toward the end of the study displayed a Ph'-containing karyotype without additional abnormalities.

and PMN counts, ³H-TdR labeling indices, as well as treatment given are included in the figures.

The fraction of blood CFC in S-phase varies between 3% and 80%, mean value 31.9 ± 14.9 (SD), when all observations (n = 60) on 12 CML patients are included. Control marrow cells from 13 hematologically normal individuals

Fig. 4. Serial studies on a 35-yr-old male (L.U.) with CML of a known duration of 2½ yr. Busulfan had previously been given April-June 1973. Hemoglobin concentration (e—e), degree of splenomegaly, neutrophil count (e—e), total leukocyte count (e—e), and treatment given is indicated. Data are also given for CFC per microliter of blood (e—e) and per cent CFC in S-phase (e—e) as well as labeling indices for myeloblasts (e—e) and promyelocytes and myelocytes (e—e). Busulfan was given at the end of this study, resulting in normalization of hemoglobin concentration, white cell count, and spleen size.
show 41.9% ± 19.5 (SD) CFC in S-phase (range 15%–80%). When data for all patients on the percentage CFC in S-phase are plotted together against WBC or PMN counts, no correlation is observed.

Figure 2 depicts serial studies carried out for 15 mo on a 63-yr-old woman with CML. During that time, when no treatment was given, an increase in leukocyte count occurred, but splenomegaly developed only after 14 months. An initial period of approximately 6 mo was characterized by a slight decrease in the percentage of blood CFC in S-phase concomitantly with the development of a slight leukocytosis. After this phase there was a rapid expansion of the S-phase fraction and a steep increase in CFC content of the peripheral blood. A period of approximately 4 mo followed when the CFC of peripheral blood as well as the CFC S-phase fraction decreased, coinciding with almost constant WBC counts, which indicated that a new equilibrium was reached. Then, however, rapid expansion of CFC concentration as well as the CFC S-phase fraction appeared concomitantly with a rapid increase of the leukocyte count and development of splenomegaly. 3H-TdR L.I. were followed during 7 mo. The L.I. of promyelocytes and myelocytes of peripheral blood increased considerably, partly coinciding with an increasing leukocytosis. Blood myeloblasts showed L.I. not higher than 15%, in accordance with earlier reports on low L.I. of myeloblasts in CML.

Serial studies of a 35-yr-old female are shown in Fig. 3. An initial phase of 5 mo was characterized by a successive increase in leukocyte count, spleen size, and CFC/μl blood. Concomitantly, the CFC S-phase fraction exhibited a continuous decrease. Busulfan treatment was then given for 4 mo with only a
moderate fall of the leukocyte count and a slight reduction in spleen size. Two months after busulfan was discontinued, the CFC S-phase fraction increased from 18% to 42%, coinciding with a rapidly rising leukocyte count. Treatment with hydroxyurea promptly normalized the leukocyte count and decreased the spleen size; the CFC S-phase fraction decreased to zero. Upon discontinuing hydroxyurea, a rebound to very high CFC and leukocyte counts rapidly occurred with an increase in CFC S-phase fraction to 45%. The close correlation between CFC concentration and WBC counts is noted.

Figure 4 shows studies of a 35-yr-old male with known CML for 2½ yr. A tendency for cyclic leukocytosis was noted with an initial periodicity of approximately 70–80 days. During the later phase, a rapid oscillating increase of the leukocytosis was seen, with a periodicity that seemed to be shorter than in the initial phase. These two phases were interrupted by busulfan treatment. From these observations, a direct correlation between the percentage of blood CFC in S-phase and the leukocyte count was drawn. The $^3$H-TdR L.I. of myeloblasts, promyelocytes, and myelocytes determined during the later phase showed no correlation with the blood CFC S-phase fraction.

Serial studies on a 41-yr-old male with CML for 27 mo are shown in Fig. 5. During a 16-mo period without treatment the leukocyte count increased slowly and splenomegaly developed. Concomitant studies of CFC, CFC S-phase and $^3$H-TdR L.I. were carried out on both peripheral blood and marrow for 12 mo. Striking parallel oscillations were found in the CFC S-phase fraction of blood and marrow with a periodicity of approximately 2 mo. A tendency for similar variations in the CFC concentration of blood was found during some part of the study. In spite of marked oscillations of the CFC S-phase cyclic variations of the leukocyte count were not apparent, although some fluctuations were indicated. The initial $^3$H-TdR L.I. showed discrepant results for blood and marrow with very low blood values. The small number of immature myeloid cells released to the blood in CML when the leukocyte count was not greatly elevated seemed to have a lower uptake of $^3$H-TdR when compared to marrow cells. It has been reported that L.I. were similar in myelocytes from blood and marrow in CML, a finding confirmed here when leukocyte counts were elevated above 50,000/μl. L.I. for myeloblasts displayed fluctuations both in blood and marrow. During some part of the study, these fluctuations were reminiscent of the CFC S-phase oscillations. The results from serial studies of this patient are also presented in Table 1. CFC, when expressed per $10^5$ A cells, exhibited large fluctuations both in blood and marrow during the course of the study and a distinct pattern was not apparent. Comparison with the data in Fig. 5 on CFC/μl blood emphasizes that CFC concentration is best expressed as CFC/μl blood. In several instances, the number of CFC/10^5 A-cells was higher in the peripheral blood than in the bone marrow, a finding also reported by others.

**DISCUSSION**

The present work has shown that CFC cycle characteristics in chronic phase CML vary greatly during the course of the disease in any given subject. This fact emphasizes the necessity for serial assays since individual determinations have little meaning. Serial studies of in vitro parameters of stem cell kinetics have not previously been reported in CML.
Table 1. Serial Studies on Colony Formation in Agar of Blood and Bone Marrow Cells From Patient C.F.5

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<th>Date of Study</th>
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*Additional patient data are found in Fig. 5.

In the chronic phase of untreated CML, the incidence of CFC in the blood is invariably greatly elevated.4,5,7,15 The present study has demonstrated a direct relationship between leukocyte counts and blood CFC concentration. We assume that CFC of blood in most cases are a representative measure of the committed stem cell pool. The unlimited growth observed in chronic-phase CML may therefore depend on expansion of the committed granulopoietic stem cell compartment. This progenitor pool is normally considered not capable of self-maintenance. The expansion observed in CML can be explained by an increased influx from the multiopotent stem cell compartment and/or an increased capacity of self-maintenance.

The existence of feedback loops regulating the production rate of granulocytes in the marrow is strongly supported by studies on irradiated animals.16,17 A granulopoiesis-inhibiting factor produced by mature human granulocytes has also been demonstrated.8,18 CML peripheral blood leukocytes can elaborate normal amounts of colony-stimulating activity (CSA).19 Therefore the great increase in leukocyte mass would point to increased production of CSA, which may lead to increased cell production in CML. A defective negative feedback or unresponsiveness to feedback regulation may also contribute. Although our work does not directly examine regulation and regulators of granulopoiesis, the finding of a decrease in the blood CFC S-phase fraction during certain phases with increasing leukocytosis (Figs. 2 and 3) is consistent with the persistence of regulatory mechanisms at this stage of the disease. With time, rapidly expanding CFC, increasing leukocytosis, and a parallel relationship between white cell counts and CFC S-phase are found. This pattern is now consistent with failure of normal regulatory mechanisms.

CML is a disease of clonal origin, as indicated by the Ph1 chromosome.20,21 The terminal acute blastic transformation is frequently associated with additional chromosomal aberrations.22,24 Clonal successions could, however, occur continuously in chronic phase CML giving rise to committed stem cells with a decreased responsiveness for growth control mechanisms and reflected in
shortening of the doubling time of WBC counts. In the course of untreated CML in patient F.K., the steep increase in S-phase (September-October, 1974 in Fig. 2) may have coincided with the introduction of a leukemic cell population with altered growth characteristics. The decreasing S-phase fraction that follows, accompanied by a stabilization of the white cell count, suggests that growth regulation is still acting. The two subsequent stepwise alterations of the S-phase fraction, which are accompanied by rapidly increasing leukocytosis, are consistent with the appearance of additional populations of leukemic cells now escaping growth control mechanisms. Similar developments can be recognized in Figs. 3 and 4. Chromosome aberrations in addition to the Ph chromosome have not been detected in our cases, but new clones might develop without alterations in chromosomal pattern. The interpretation of rapid alterations in CFC S-phase as signals for new cell populations is, however, so far speculative.

The existence of cyclic leukocytosis in CML may be taken as evidence for retained responsiveness to control mechanisms. The extended period in cyclical CML has been explained by increased maturation time and, consequently, a time delay in the negative feedback control. The present results do not, however, indicate that a negative feedback mechanism elaborated by the mature granulocytes is involved, since an inverse relationship would have been expected between leukocyte count and CFC S-phase fraction as found in cyclic neutropenia. Data in Fig. 4 indicate a direct relationship. The marked periodicity of the CFC S-phase fraction in one patient (Fig. 5) without distinct cycles in the leukocyte count is a unique observation. Negative feedback control by mature granulocytes is not likely to be involved. Although not proved by our data, the cyclic variations in CFC S-phase of this case are consistent with cyclic activity in the multipotential stem cell compartment. Alternatively, a negative feedback mechanism elaborated by a cell other than the mature granulocyte could be proposed as an explanation for CFC S-phase cycling.

The lack of correlation between 3H-TdR labeling indices and percentage of CFC in S-phase indicates that there are changing relationships between committed granulopoietic stem cells and differentiated cell kinetics during chronic phase CML. The cause of these alterations is obscure. They are, however, consistent with several mechanisms of autoregulation. Stem cells and differentiated cells may vary in susceptibility to growth regulation. Further studies of granulopoietic regulation in CML may shed light on the mechanisms involved in blast cell transformation.

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Granulopoiesis in chronic myeloid leukemia. II. serial cloning of blood and bone marrow cells in agar culture

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