Alterations of Granulopoiesis Following Chemotherapy

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Clonal proliferation of marrow granulocytic progenitor cells (GPC) in vitro and the daily urinary output of granulocytic colony-stimulating factor (CSF) were determined in two patients with acute myeloid leukemia (AML) in remission and one patient with malignant lymphoma receiving monthly pulses of chemotherapy with cytosine arabinoside and 6-thioguanine. During and immediately following therapy, a marked decrease of granulocytic colony-forming capacity (CFC) occurred, with an increase and return to basal levels by 3–4 wk. In the AML patients, the proportion of GPC in DNA synthesis (GPC-S), as determined by the thymidine suicide technique, declined from basal levels (31%–39%) to 0%–26% after 2 days of treatment. This was followed by a sharp rise in GPC-S to 41%–75% 1–3 days posttherapy, with an oscillatory return of GPC-S to basal levels by 3–4 wk. In all courses a marked increase of urinary CSF output occurred during or 1 day posttherapy, concomitant with the rise of the proportion of GPC-S. In the lymphoma patient, an initially high proportion of marrow GPC-S and low CFC anticipated the severe neutropenia which followed therapy. These results provide a basis for determining the efficacy with which cytotoxic drugs destroy proliferative activity of GPC and for assessing the potential for hemopoietic toxicity following chemotherapy.

A CENTRAL FEATURE FOR improving chemotherapeutic programs directed at malignancy is the necessity of maximizing tumor effect and minimizing toxicity to normal hemopoietic cells. More rational treatment regimens could be devised if the susceptibility of normal granulopoietic precursors to chemotherapeutic agents was known. Recent investigations utilizing marrow culture techniques have improved our understanding of factors affecting the regulation of granulopoiesis in animals and man. These quantitative in vitro methods determine the clonal proliferation and differentiation of marrow granulocytic progenitor cells (GPC) in agar under the necessary influence of the humoral stimulatory substance termed colony-stimulating factor (CSF).

Following irradiation and cytotoxic drug exposure of mice, the granulocytic colony-forming capacity (CFC) is altered, and serum CSF levels are increased. Irradiation and endotoxin stimulate marrow regeneration and induce marked increases of the proportion of murine GPC in DNA synthesis (GPC-S). The sensitivity of mouse hemopoietic stem cells and GPC to

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chemotherapeutic agents is dependent on their proliferative state, with marrows which contain a high proportion of GPC-S being more susceptible to the toxic effects of these drugs.\(^{11}\) We have characterized alterations of marrow CFC and GPC-S and urine CSF output in patients undergoing pulse chemotherapy with cytosine arabinoside (Ara-C) and 6-thioguanine in order to assess the effects such perturbations have on human marrow granulocytic proliferative activity and to determine whether changes in these parameters can predict hemopoietic toxicity.

**MATERIALS AND METHODS**

**Human Marrow Proliferative Activity**

Our methods for performing the in vitro assays for human marrow CFC and the proportion of GPC-S have previously been described.\(^{3,12}\) With the thymidine suicide technique, the difference between the number of colonies formed from the cell suspension exposed to high-specific-activity tritiated thymidine (16 Ci/mM, Schwartz-Mann) relative to the number formed in the non-exposed control suspension indicates that proportion of GPC which were in S phase during exposure. Only those GPC in S phase of the cell cycle had their proliferative capacity inhibited; thus, the percentage expressed represents a lower limit of the actual proportion of cells in cycle. Shifts in the fraction of cells killed by thymidine suicide reflect an alteration in the relationship of the duration of DNA synthesis to the total generation time, a change in the growth fraction, or induced partial synchronization of the cell population. Previous studies by other workers indicate that alterations of exogenous thymidine incorporation into DNA following irradiation or Ara-C exposure were not due to changes in either intracellular thymidine pool size\(^{13}\) or thymidine kinase activity.\(^{14,15}\) As previously reported,\(^{3}\) our range for control marrow CFC is 14-36 colonies per 10\(^5\) nucleated marrow cells plated (26 ± 1, mean ± SE), and for the proportion of GPC-S it is 31%-39% (35 ± 1, mean ± SE).

**Urinary Output of Colony-stimulating Factor (CSF)**

For evaluating the daily urinary output of CSF we have used the calcium phosphate adsorption and concentration technique, reported by Stanley et al.,\(^{16}\) which permits quantitation of urine CSF essentially free of inhibitory substances. Twenty-four hour urine specimens were collected in polypropylene or glass containers with 0.02% sodium azide preservative. Thirty-six milliliter aliquots of the measured urine samples were dialyzed for 24 hr at 4°C against three changes of 20 volumes of distilled water, and 1 ml was saved for protein determination.\(^{17}\) The dialyzed urine was mixed with 2 ml of calcium phosphate gel in 0.01 M phosphate buffer, pH 7.4 (BioRad Laboratories, Richmond, Calif.). This mixture was centrifuged at 600 g for 7 min at 4°C, the supernatant discarded, and the gel pellet resuspended with 4 ml of 0.03 M phosphate buffer, pH 7.4. The suspension was then recentrifuged, and CSF was recovered from the eluted supernatant. This material was Millipore filtered, stored at 4°C, and bioassayed within 2 mo of collection.

The bioassay used for quantitating human urine CSF has been previously described.\(^{7}\) Duplicate 0.15-ml urine samples were used to stimulate mouse marrow granulocyte-macrophage colony growth in tissue culture dishes containing 7.5 x 10\(^4\) marrow cells from 2-3 mo-old male C57Bl mice in 1 ml of modified McCoy’s 5A-0.3% agar medium. Colonies containing more than 50 cells after 10 days of humidified incubation in 7.5% CO\(_2\) in air were counted, and most colonies consisted of 250-1500 cells. All specimens were tested undiluted and in serial aqueous dilutions of 0.75 x, 0.5 x, and 0.25 x. On certain specimens, larger amounts or lower concentrations of urine were also plated. No decrements of colony-stimulating activity were noted when increasing concentrations of stimulatory specimens were employed, suggesting the absence of inhibitory materials in these samples. Titration curves permitted quantitative estimates of effective CSF concentrations. Pretreatment urine specimens were assayed at the same time as the urine specimens for each monthly course of therapy.

The following equation, based on that utilized by Stanley et al.,\(^{16}\) was used to determine urinary CSF output:
CSF units/day = \frac{6.66 \cdot a_u \cdot \text{volume}_u \cdot 1x_s}{C.F. \cdot a_s}

A unit of CSF has been defined as that amount necessary for stimulating one colony in vitro. The concentration factor (C.F.), which takes into consideration the amount of liquid from the calcium phosphate gel in the final eluate, was 6.85 for most of the experiments. \(a_u\) and \(a_s\) represent the computer-calculated effective CSF concentration of the unknown test urine and the standard urine, respectively. \(1x_s\) represents the number of colonies stimulated by the undiluted 0.15-mI standard urine. Multiplication by 6.66 converts CSF from 0.15 ml to 1 ml, and multiplying this product by the 24-hr volume (in milliliters) of the unknown urine (termed \(\text{volume}_u\)) indicates the daily CSF output. Urine CSF specific activity was evaluated by expressing daily CSF output relative to protein excretion. Maritz et al.\(^{18}\) have described a model for analyzing results of CSF-marrow target cell interaction. We have utilized curve-fitting computer programs (prepared by Dr. Rupert Miller and Ann Varady, M.S., Stanford University Department of Biostatistics) based on this model to determine the effective concentration of CSF (termed \(a\)) from the titration curves of the test urines as compared to similarly prepared stable normal urine standards. Control values for CSF output and CSF output relative to protein, obtained from 11 normal adults aged 22-58 yr., were 68,000 \(\pm\) 21,000 U (mean \(\pm\) SD) excreted per day (range, 36,000-97,000) and 191 \(\pm\) 21 CSF U per mg protein (range, 124-291), respectively. Twenty-seven urine specimens containing 60,000-80,000 U of CSF stimulated 31-138 colonies with 0.15 ml of undiluted processed urine, indicating the need for performing titration curves in order to appropriately rank the CSF content of specimens.

**Patient Selection**

The study group was comprised of two patients (B.K., 32M; T.F., 42F) with acute myeloid leukemia (AML) in remission, and a patient (R.H., 67M) with malignant lymphoma, diffuse histiocytic type, Stage IVB, without marrow involvement by tumor. The AML patients in remission received monthly 2-day maintenance courses of Ara-C, 150 mg/sq m/day (3.6 mg/kg/day), given as 4-hr intravenous infusions of 75 mg/sq m every 12 hr (four doses), and 6-thioguanine, 2.5 mg/kg/day, given orally. The lymphoma patient was treated with 5-day courses of adriamycin, 60 mg/sq m intravenously on day 1, and Ara-C, 3 mg/kg intravenously and 6-thioguanine, 2.5 mg/kg orally on days 2-5. From 8 to 16 hr were permitted to lapse after the end of treatment before performing posttreatment marrow aspirations for study of in vitro marrow proliferative activity. Serial complete blood counts and serum muramidase values\(^{19}\) were also determined in these patients.

The protocol for this investigation was approved by the Stanford University Medical Center's Human Subjects Committee, and the implications and risks of the study were discussed in detail with the patients.

**RESULTS**

Marrow CFC and the proportion of GPC-S were evaluated for two AML patients in remission (B.K. and T.F.) undergoing chemotherapy. A preliminary report of part of the study of B.K. has appeared.\(^{20}\) Composite values of 53 CFC determinations obtained at various times over 20 courses of therapy for these patients are shown in Fig. 1A. The curves in the figure were fitted by visual inspection. A marked decrease to approximately one-third of the basal median CFC value occurred after 1–2 days of therapy. A subsequent gradual increase occurred over the next week, with a return to basal levels by 3–4 wk. Our data do not permit extrapolation of the CFC pattern between days 14 and 24.

In Fig. 1B are shown the composite values of 34 determinations of the proportion of GPC-S obtained over 11 courses of therapy. These data indicate that a decrease from basal levels to generally 0%–20% GPC-S was present 8–16 hr after 2 days of therapy, followed by a marked increase to 41%–75% of GPC-S.
Fig. 1. Composite data of (A) marrow colony-forming capacity and (B) the proportion of granulocytic progenitor cells in DNA synthesis following cytosine arabinoside (Ara-C) and 6-thioguanine (6-TG) treatment of two patients with acute myeloid leukemia in remission (B.K., T.F.). Curves were fitted by visual inspection.

Fig. 2. Concomitant serial alterations of marrow colony-forming capacity and the proportion of granulocytic progenitor cells in DNA synthesis (± SE) following cytosine arabinoside (Ara-C) and 6-thioguanine (6-TG) therapy of two patients with acute myeloid leukemia in remission (B.K., T.F.). Dark rectangles denote periods of treatment.
1–3 days after completion of treatment, with an oscillatory return to basal levels by 3–4 wk.

Figure 2 shows the results of five courses in which sequential CFC and GPC-S evaluation performed concomitantly included at least four determinations. A reciprocal relationship between the initial decrease of CFC and increase of GPC-S 1–2 days posttherapy was noted in courses 2, 3, and 4. This pattern of marrow response was also indicated by the composite data in Fig. 1. On several occasions, when too few cells were obtained, CFC but not thymidine suicide studies were performed.

Sequential values of daily urinary CSF output determined throughout seven courses of therapy are shown in Fig. 3. In three courses a decrease in the observed CSF output occurred on day 1; however, in all courses marked increases (median, 256%) were noted by days 2–3 (i.e., on the day of completion of therapy and 1 day after this). A fall of CSF output into the normal range then occurred over the next few days, with moderate fluctuations persisting during the following month. Parallel changes of urinary CSF specific activity relative to protein excretion (Fig. 3) indicated that alterations in CSF values were not due to nonspecific generalized variation of total protein excretion. The urinary
CSF values of courses B and C (Fig. 3) were obtained in the same courses as the marrow CFC and GPC-S data of courses 2 and 3, respectively (Fig. 2). These data show that the rise in the proportion of GPC-S occurred concomitant with elevation of urinary CSF output.

Serum muramidase values (42 determinations in B.K. during eight courses) obtained throughout the period of observation ranged from 18 to 42 µg/ml (normal, 15-45 µg/ml), and no consistent alteration of these levels was noted in relation to therapy.

Throughout the study, B.K. did not develop neutropenia, whereas moderate transient neutropenia was noted 3 wk after treatments in T.F., a patient with a relatively short remission period.

The patient with lymphoma (R.H.) had a slightly hypocellular marrow without tumor infiltration the day prior to therapy. At that time his marrow CFC was low with a high proportion of GPC-S (Fig. 4). After completion of the course of therapy, CFC decreased acutely, followed by a rise 8 days later coincident with a severe neutropenia and preceding the subsequent neutrophil elevation. Prior to his second course of therapy, marrow CFC and proportion of GPC-S were normal, and his subsequent neutrophil nadir was less marked (Fig. 4).

**DISCUSSION**

Our studies have shown that marked quantitative alterations of CFC, GPC-S, and urinary output of CSF occurred in patients undergoing pulse chemotherapy. Thus, when using these measurements for determining the clinical status of patients, it is necessary to consider treatment as a possible
cause for changes which become evident in these parameters over the following month. Since in vitro growth patterns described in our previous study and cytogenetic analyses by Moore and Metcalf indicated that colonies from marrow of patients with AML in remission were derived from remaining normal GPC, the CFC and GPC-S values monitor proliferative changes occurring within the population of normal granulopoietic cell clones.

In our patients with AML and lymphoma, a marked decrease in the concentration of marrow CFC occurred within 1–2 days after the onset of treatment, with an increase and return to basal levels by 3–4 wk. These findings are similar to studies in mice wherein CFC and the size of the GPC compartment decreased initially, with oscillatory recovery of granulopoiesis occurring following exposure to cytotoxic drugs. A similar pattern of CFC alteration in lymphoma patients following chemotherapy has also been shown by other workers. It should be emphasized that in man CFC data represent the concentration of GPC in a given marrow sample. Variation in marrow differential counts, dilution by peripheral blood, and differing myeloid marrow distribution patterns affect this measurement. Loss of GPC by differentiation or migration also diminishes the ability to quantitate granulopoiesis by CFC evaluation alone. Therefore, the decreased CFC values could have resulted from qualitative changes in marrow cell composition and/or the anticipated decrease in total CFC due to cytotoxic chemotherapy.

The thymidine suicide technique, which reflects the proliferative state of the GPC by determining the proportion of GPC-S, appears to provide a more representative measure of marrow granulopoietic responsiveness, since results of these studies are not altered by the above-mentioned factors. A decrease of the proportion of marrow GPC-S occurred during and for 8–16 hr after treatment, followed by a marked increase and then an oscillatory return to basal levels by 3–4 wk. The acute decrease of GPC-S most likely resulted from the S-phase arrest or killing induced by Ara-C and 6-thioguanine on the proliferative activity of susceptible GPC (i.e., GPC-S). The subsequent overshoot of the proportion of GPC-S, which occurred within 1–2 days following completion of therapy, appeared to be due to partial synchronization by drug therapy of surviving proliferative GPC. A similar pattern of leukemic cellular kinetics following Ara-C therapy has been attributed to synchronization and recruitment of resting cells into cycle. Our results indicated that posttreatment marrow CFC concentration was low when the proportion of GPC-S was increased. These findings are consistent with previous data using mouse marrow indicating that decreased marrow cellularity occurred concomitant with increased proportions of hemopoietic stem cells, GPC, and more differentiated granulocytic precursors in DNA synthesis.

Blood neutrophil levels remained normal during treatment, indicating that in man, as in mice, neutropenia does not cause the early increases of CSF output following exposure to cytotoxic agents. No systematic variations in serum muramidase levels were found in our patients; thus, our data do not suggest utility of muramidase determinations for monitoring granulopoiesis.

Marked elevations of urinary CSF output occurred acutely following chemotherapy, with a subsequent fluctuating return to basal levels by 3–4 wk. No such
variations are present in untreated individuals. Increments in the proportion of marrow GPC-S occurred in parallel with increased urinary CSF output in our patients. Similar increments occur in mice concomitant with increases in serum CSF levels. Incubation of hemopoietic cells with CSF in vitro causes an increased proportion of GPC-S. Recent investigations from our laboratory have shown urinary CSF output to correlate with parallel changes of marrow CFC and GPC-S. However, since human urinary CSF stimulates mouse but not human marrow cell proliferation, the physiologic meaning of the relationship between urinary CSF and marrow proliferative changes remains to be clarified.

Severe neutropenia following therapy occurred only in the lymphoma patient, and in that patient when his marrow CFC was low and the proportion of GPC-S was high prior to treatment, but not when these values had returned to normal. Recovery of his peripheral neutrophil counts occurred subsequent to increased marrow CFC and GPC-S. These findings confirm data obtained with actively proliferating mouse marrow indicating that the increased proportion of GPC-S detected by thymidine suicide reflects enhanced in vivo sensitivity of granulocytic precursors to cycle-active chemotherapeutic agents. Detecting granulopoietic compensation of regenerating marrow proliferative status thus appears to provide a useful index for anticipating potential drug-induced neutropenia in man. Design of chemotherapeutic regimens with this type of analysis should help to diminish granulopoietic toxicity.

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