Factors in Human Serum Affecting the Proliferation of Normal and Leukemic Cells

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SUMMARY

Serum factor(s) regulate the rate of proliferation of normal and leukemic bone marrow cells. Nine patients with solid tumors and normal marrow function were treated with cyclophosphamide (60.0 mg/kg) on each of 2 successive days. Following aplasia, the granulocytic proliferative fraction of the patients' marrow increased twofold (tritiated thymidine autoradiography). With maturation of the marrow elements, the tritiated thymidine indices fell below pretreatment values. Serial serum samples obtained from these patients affected the incorporation of tritiated thymidine into DNA in primary cultures of normal bone marrows. In relation to pretreatment sera, samples obtained during the proliferative phase caused a twofold increase in tritiated thymidine incorporation by target cells, and sera obtained during the marrow maturation phase caused a twofold decrease. Identical results were obtained when these sera were incubated with marrows from patients with acute lymphoblastic and acute myelocytic leukemia (> 95% tumor) and when they were assayed with the long-term cultured cell lines.

Thus, factor(s) are present in these sera that can affect tritiated thymidine uptake in both normal and leukemic hematopoietic cells.

INTRODUCTION

The periodic oscillations of bone marrow homeostasis are accentuated by the depletion of peripheral blood cells (5) or the suppression of bone marrow proliferation by irradiation (10) or cytotoxic drugs (3, 9). Cellular aplasia is followed by recovery and overshoot. The oscillations in peripheral blood cell concentrations following these perturbations suggest the presence of a negative feedback inhibition of bone marrow proliferation.

By means of a simple assay, we have detected substance(s) in human sera obtained during the period of CY3-induced bone marrow aplasia and recovery that influence proliferation in a variety of cell types.

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3 The abbreviations used are: CY, cyclophosphamide; TdR-3H, tritiated thymidine; L.I., labeling indices; LTTC, long-term suspension-cell cultures.

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MATERIALS AND METHODS

Patient Selection and Therapy. The patients tested had progressive noncurable neoplasms but had no detectable physiological deficits. In particular, hematopoietic function was adjudged normal by peripheral blood examination, bone marrow biopsy, and aspiration. No marrow-depressing therapy had been administered within 4 weeks of this study. All of the patients received CY (kindly provided by Dr. Paul Worrall of the Mead Johnson Co., Evansville, Ind.), 120 mg/kg, given either in 2 equally divided doses at 24-hr intervals, or by continuous 48-hr infusion.

Preparation of Microautoradiographs. Aliquots of aspirated bone marrow cells obtained prior to therapy and at intervals thereafter (until peripheral white blood cell count stabilization), were added to 5 ml of heparinized tissue culture media (Roswell Park Memorial Institute Medium 1640, 15% calf serum) containing TdR-3H, 0.1 µCi/ml. After 1 hr of incubation at 37° in a 7% CO2 atmosphere, the cells were washed and concentrated by light centrifugation (relative centrifugal force, 42 X 15 min). Smears of the pellet were made on slides coated with gelatin. Autoradiographs were prepared with Kodak NTB-2 photographic emulsion exposed for 21 days, developed, and stained with Giemsa. The TdR-3H L.I. were determined by the number of cells per 1000 counted that contained 5 or more grains overlying the nucleus. Results are reported as percentage labeled.

Test Sera. Prior to therapy and 3 times a week until the peripheral white blood cell count stabilized, 10 ml of whole blood were collected from each patient and allowed to clot, and the sera were separated sterilely and stored immediately at −70°. All sera were then analyzed simultaneously.

Target Cell Suspensions. Cells from established LTTC derived from the peripheral blood cells of patients with acute lymphoblastic leukemia were suspended in Roswell Park Memorial Institute Medium 1640. Aspirated samples of normal and acute leukemic bone marrow were similarly handled. We obtained a single-cell suspension by drawing it through a 25-gauge needle. Fibroblasts were derived from skin explants of patients and from human foreskin culture. One-ml aliquots of the target cell suspension at a concentration of 1 X 10⁶/ml were added to Falcon 3303 culture tubes, and the recently thawed sera to be analyzed were added to triplicate tubes to a concentration of 15%.

TdR-3H Incorporation Assay. This method is analogous to the soft agar assay of conditioning factor activity (1). In all assays, 1.0 µCi of TdR-3H (specific activity, 1.9 Ci/m mole) was added to each tube of media and incubated with the test

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cells at 37° for 18 hr (42- and 62-hr cultures gave similar results). The cultures were terminated by immersion in ice, and the contents of each tube (10⁶ cells) were collected under vacuum on a 934AH glass fiber filter disc (H. Reeve Angel & Co., Inc., Clifton, N. J.). Each tube was washed with cold 0.9% NaCl solution, and the wash was added to the filter. Following 3 washings with 0.9% NaCl solution, the acid-insoluble materials on the filter were precipitated with 3 washings with cold 5% trichloroacetic acid and then were washed 3 times with absolute ethanol. The dry filter was transferred to screw-top vials to which 6 ml of Bachs scintillation fluid were then added, cooled, and counted in a liquid scintillation spectrometer. Results are expressed as cpm/tube X 10⁴. During the brief period of culture, the numbers of test cells and their viability (as determined by trypan dye exclusion) remained constant.

RESULTS

Peripheral Blood. The counts of the peripheral blood of 9 patients who received CY are depicted (Chart 1). Subsequent to therapy, the WBC was maintained until Day 4, at which time a precipitous decrease occurred and granulocytopenia ensued. Granulocytic elements were again detected by Day 14; they increased to a zenith on Day 32 and then declined to pretreatment levels by Day 36. The platelet count nadir occurred on Day 12, with a rebound paralleling the WBC. Reticulocyte release ceased on Day 6 and reappeared on Day 12.

Bone Marrow. The morphology of serial bone marrow aspirates from each patient was reviewed (Chart 1). In general, aplasia occurred by Day 3. Recovery began on Day 6. Cohorts of granulocyte precursors progressed through the proliferative (myeloblasts, progranulocytes, early myelocytes, Days 6 to 12), and maturative (late myelocytes, metamyelocytes, bands, and segmented neutrophils, Days 13 to 22) compartments. During a brief "hypermature" stage, the marrow contained primarily segmented neutrophils with very few immature elements (Days 23 to 28). Megakaryocytes and erythroid precursors were noted by Day 8.

Changes in Bone Marrow Proliferation. The TdR-³H L.I. of the granulocytic elements of bone marrow were determined on bone marrow specimens obtained serially from 6 patients (Chart 2). Cells labeled by this flash method are those involved in proliferation, i.e., myeloblasts, progranulocytes, and early myelocytes. It is assumed that the duration of the phase of

![Chart 1. The changes in peripheral blood counts of 9 patients given CY. ●, WBC; X, platelet count; ○, reticulocyte percentage; vertical lines, S.E. Bone marrow aspirates were classified according to the predominant granulocytic cell type present.](chart1.png)
DNA synthesis does not vary. Changes in the L.I. followed a pattern similar to changes in the effect of patients' sera on TdR-3H uptake by target cells (Chart 4).

**Serum Studies from a Single Patient.** When compared to pretreatment studies, sera obtained from 1 patient immediately after CY administration caused a decrease in TdR-3H uptake by the target cells (LTTC). Cytotoxic CY metabolites were present in these sera (Chart 3). The half-life of nitrobenzylpyridine (alkylating) metabolites of CY in the serum is 6 hr after cessation of drug. (O. M. Colvin, personal communication).

Subsequent posttreatment sera produced a progressive increase in TdR-3H uptake by target cells, compared with pretreatment studies. Peak values were observed from sera obtained when progranulocytes and myelocytes predominated in the marrow. However, sera obtained during the maturative bone marrow phase and the subsequent peripheral WBC recovery stage inhibited TdR-3H uptake.

Autoradiographic analyses of bone marrow aspirates revealed analogous changes in L.I. of the granulocytic elements. The indices observed during the proliferative phase of recovery were higher and those sera during the hypermature stage were lower than the TdR-3H L.I. of the marrow before CY administration.

**Changes in Serum Activity.** Nine patients who received CY were studied (Chart 4). In 1 patient, no change was detected in the effect of serial serum samples on TdR-3H uptake by target cells (LTTC). Sera obtained from 8 patients from the 3rd to 14th day after CY administration enhanced the uptake of TdR-3H compared with results with pretreatment sera. Sera obtained from 5 of these patients on Days 18 to 38 reduced TdR-3H uptake. One patient was not followed through WBC recovery. In 4 of these 5 patients, apparent inhibition of TdR-3H uptake was shown after the serum concentration in the assay system was increased from 15 to 30%. Concurrent masking of the stimulatory activity suggests a dose response to an inhibitor. The duration, slope, and temporal relationships of the DNA synthesis activity excludes mediation by casual endotoxin contamination (3).

Sera from 2 patients failed to depress this TdR-3H uptake, in comparison to pretreatment sera. Subsequent to peripheral WBC recovery, both patients revealed persistent and marked
neutrophilia, with counts >25,000 cu mm. At autopsy, each patient had widely disseminated neoplasm with bone marrow infiltration not detected by pretreatment studies.

**Specificity of Serum Effect.** To determine specificity of activity of these serum factor(s), we used a variety of cell types and completed simultaneous assays in the presence of the same sequential sera at a concentration of 15% (Chart 5). The uptake of TdR-3H by normal bone marrow, bone marrow cells from a variety of human acute leukemias with >95% leukemic cells, human fibroblasts, long-term cell lines derived from patients with acute lymphoblastic and myeloblastic leukemia, normal rat bone marrow, and mouse L1210 leukemia was affected similarly by all sera. Thus, the serum-mediated effect on TdR-3H uptake was nonspecific with respect to cell type and species of origin. It is of particular interest that human leukemia responds to these regulators.

Sufficient sera were available from 5 patients to permit examination regarding the nature of the active substances. The proliferative capacity of 3 replicate target cell cultures containing pretreatment sera, peak noninhibitory sera obtained at about the WBC nadir, and markedly inhibitory sera obtained at about the recovery of the peripheral WBC to pretreatment values were compared with controls that lacked serum. The controls and the sera obtained during the WBC nadir similarly facilitated TdR-3H uptake into target cells, compared to normal pretreatment sera (Chart 6). These data are consistent with the presence of an inhibitor(s) in the pretreatment sera. This inhibitor(s) decreases during the period of CY-induced aplasia, is not detectable during the proliferative phase of bone marrow recovery, reappears, and attains maximal concentration during the maturing phase of marrow recovery and subsequent peripheral WBC recovery.

**DISCUSSION**

These studies showed that there are factor(s) present in human serum which affect cell uptake of TdR-3H during an 18-hr in vitro cell culture. Following a large, myelosuppressive dose of the alkylating agent CY, serum samples at first appear to facilitate and later restrict the cell uptake of TdR-3H in comparison with the effect of pretreatment sera. The L.I. of the granulocytic elements of the recovery bone marrow are similar temporally. Whether these phenomena are causally related is not clear.

The precise identity of the factor(s) that regulate granulocyte homeostasis and their source(s) are unknown. The rate of

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**Chart 3.** Changes in the WBC of a patient given CY, 120 mg/kg. •¿, times of blood drawing for CBC and serum collection. These serial sera affected the TdR-3H incorporation into target cells (●). All TdR-3H uptakes were measured simultaneously, and the means of 3 replicate determinations were plotted ± S.E. Actual data in cpm are presented to demonstrate the range of error of the method. The TdR-3H L.I. of the granulocyte elements of the bone marrow are expressed as percentage of cells labeled (X). Changes in marrow morphology were classified according to the predominant granulocytic cell present. Vertical lines, S.E.; SEC, segment.
production of granulocytes by the bone marrow seems to oscillate in response to the peripheral demand for these cells. Morley (6) detected oscillations lasting 14 to 24 days in the peripheral granulocyte counts of healthy humans. Oscillations of greater amplitude but similar duration have been observed in laboratory animals and humans given cytotoxic drugs (3, 9, 10).

The post-CY oscillations in peripheral granulocyte counts suggest the operation of a negative feedback inhibitor (7). This presence of a regulator in the peripheral blood, possibly related to the circulating granulocyte, has long been suspected (on the basis of indirect evidence (2, 4, 11–13). Our data suggest the presence of an inhibitor or, alternately, of both stimulator and inhibitor substances in variable amounts which occur simultaneously with granulocytic proliferation. The increase in target cell TdR-\(^3\)H uptake may reflect a lessening of the overall inhibitory activity normally present in sera. This inhibitory activity then returns to the serum during the maturing phase of the bone marrow recovery when the predominant granulocytic cell is a late myelocyte, and it persists through the hypermature stage until the peripheral granulocyte count has normalized. Previous analogous assay methods (soft agar, spleen colony (1)) have not permitted definition of this variable inhibitory activity in serum during marrow recovery from cytotoxic agents. That the maturing granulocyte element of the bone marrow may emit this regulator is reminiscent of the theories of Rytomaa and Kiviniema (14) and of Osgood (11) regarding a tissue-specific self-regulator. However, it is questionable whether the mature peripheral blood granulocyte produces an inhibitory regulator, since quite the opposite activity has recently been attributed to it (12). In support of this, 2 of our patients demonstrated marked neutrophilia without detectable serum-inhibitory activity.

The early serum-mediated changes in TdR-\(^3\)H uptake are reminiscent of the observations of Morley and Stohlman (8). They found that serum obtained from irradiated rats during the early period of marrow recovery enhanced the granulocyte colony-forming activity of normal marrow (soft agar assay system). Their findings suggested the presence of a granulopoietin. However, their assay system permits the growth of only granulocytic and monocytic colonies. As Morley and Stohlman themselves cautioned, the damage caused by whole-body irradiation and cytotoxic drugs affects tissues (especially those with some degree of proliferative activity), in addition to the hematopoietic elements of the marrow. There is no conclusive evidence that the regulators are cell-type specific, and extramedullary sources of these factor(s) cannot be excluded.

Our observations indicate that the serum factor(s) that
Chart 5. Various target cells including human normal and neoplastic bone marrow, fibroblasts, and mouse L1210 leukemia incubated with the sera of 1 patient showed a similar pattern of TdR-3H uptake.
Chart 6. Sera of 5 patients (each represented by a symbol) that affected the proliferation of target cells. The incorporation of TdR-3H by cells incubated with sera obtained prior to therapy (A), with peak noninhibitory sera obtained at about the nadir of the peripheral WBC (B), and with markedly inhibitory sera obtained about the peripheral WBC recovery to pretreatment values (C) was compared with that of cells incubated without sera (No Serum Control). The No Serum Control is similar to B and suggests that the normal regulatory factor(s) present in serum are inhibitory.

Affect TdR-3H uptake in our assay systems are nonspecific with respect to the type of cell affected or the species of origin of the cell. Thus, multiple factors may be present, or there may be 1 factor which is catholic in its activity. The effect of these factor(s) on leukemic cells suggests that humoral factors may be involved in mediating the proliferative kinetics of hematopoietic neoplasia.

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