

Influence of Humoral Regulators on Proliferation and Maturation of Normal and Leukemic Cells¹

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SUMMARY

Factors that influence the proliferation of marrow elements can be detected in sera. To determine the function and to compare the effect of these factors, cells were obtained from patients with normal and leukemic bone marrows. The effects of drug-induced stimulatory and inhibitory sera and leukemic pretreatment sera over time (0 to 6 days) on proliferation and granulocytic morphology of normal and leukemic bone marrow cells in culture were evaluated. Increased proliferation was associated with stimulatory sera, while inhibitory and leukemic pretreatment sera retarded proliferation of both normal and malignant cells. Exposure of normal proliferative cells to inhibitory or leukemic pretreatment sera yielded the greatest increase in mature granulocyte forms. In contrast, although the proliferative response of leukemic cells to these sera was similar to normal, maturation was minimal. These data suggest that leukemic pretreatment sera are similar to inhibitory sera and are not leukemogenic. Both retard proliferation of normal and leukemic bone marrow cells while enhancing maturation of normal cells. Leukemic myeloblasts, however, cannot be made to mature by these humoral regulators.

INTRODUCTION

The oscillatory nature of hematopoiesis can be amplified by disease and by the induction of bone marrow aplasia (1, 5, 7, 9, 12, 20, 21, 24, 27). These exaggerated oscillations of circulating blood cells and their bone marrow precursors relate inversely to measurable humoral factors (1, 3, 7, 21, 27). Serum obtained at the time of bone marrow proliferation following myelosuppression increases [³H]TdR³ incorporation by cultured bone marrow cells, while serum obtained coincident with the nonproliferative marrow recovery phase inhibits [³H]TdR incorporation (1-3). The temporal association of the effects of these stimulatory and inhibitory sera with a specific phase of bone marrow recovery suggests a direct relationship between serum factors and the growth of marrow cells (15). If so, it is plausible that imbalance

of these humoral factors, a premise examined in *in vitro* systems, could be involved in the leukemic process (6, 16, 17, 22).

Using sera obtained from myelosuppressed individuals with normal bone marrow and from untreated leukemic patients, we have examined the effects of these sera on the proliferation of normal and leukemic bone marrow cells in an *in vitro* system. Further, using the granulocytic cell series as our morphological monitor of marrow development, we have examined the effects of these sera on the maturation potential of normal and leukemic cells.

MATERIALS AND METHODS

Bone Marrow Cells

Bone marrow cells were obtained from 11 patients by routine needle aspiration over a 7-month period. Four patients had normal marrows before myelosuppressive therapy and 7 patients had acute leukemia.

Normal Proliferative Cells. Early proliferative phase recovery bone marrow cells were obtained from 4 patients recovering from drug-induced aplasia during therapy for solid tumor. These recovering marrows contained a >70% proliferative granulocyte cohort, had a myeloid:erythroid ratio of 6 to 7:1, and were without evidence of marrow involvement by tumor. The [³H]TdR LI of these proliferative phase bone marrow cells has previously been demonstrated to be 200 to 600% of pretreatment LI (1).

AMMOL Cells. Bone marrow cells were aspirated from 3 patients with AMMOL [as described by Saarni and Linman (26)] prior to any chemotherapy.

AML Cells. Bone marrow cells were aspirated from 4 patients with AML whose marrows contained >95% leukemic cells prior to any chemotherapy. All marrow samples were obtained at a time when patients were clinically free of all evidence of infection and bleeding. Cells were collected in Roswell Park Memorial Institute Medium 1640 and immediately prepared as a single cell suspension by being repeatedly drawn through a 25-gauge needle. For clarity, we have separated granulocytic cells into morphological compartments, which are referred to as proliferative (myeloblast, progranulocyte, myelocyte) and nonproliferative (metamyelocyte, band, segmented neutrophil).

Specific Sera Collection

Sera were obtained by plasmapheresis from 5 patients with localized solid tumor without bone marrow involve-

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³ The abbreviations used are: [³H]TdR, tritiated thymidine; LI, labeling index; CY, cyclophosphamide; AMMOL, acute myelomonocytic leukemia; AML, acute myelocytic leukemia; NCC, nucleated cell count.

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ment undergoing induced bone marrow aplasia. These patients had progressive noncurable neoplasms, but hematopoietic function was adjudged normal by peripheral blood examination, bone marrow biopsy, and aspirate. No marrow-depressing therapy had been administered within 4 weeks of this study. All 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. Sera were collected and prepared as described previously (1). All patients had pretreatment sera that affected [³H]TdR incorporation by bone marrow cells within 1 S.E. of the range obtained with 30 normal sera. To obtain adequate amounts of sera, 3 to 6 individual sera/patient, which either stimulated or inhibited [³H]TdR incorporation by bone marrow cells, were selected and pooled. Maximally stimulatory sera occurred on Days 6 to 14 of high dose CY therapy and ranged from 135 to 200% of pretreatment [³H]TdR incorporation. Inhibitory sera were obtained on Days 21 to 35 of CY therapy and ranged from 35 to 75% of pretreatment [³H]TdR incorporation. Leukemic pretreatment sera were obtained from the 7 patients with AMMOL or AML whose bone marrow cells were studied. Normal serum was obtained from a volunteer (P. J. B.) whose sera, on repeated assay, have been within 1 S.E. of the range obtained with 30 sera from other normal individuals.

Cell Cultures

Bone marrow cells in a total volume of 1 ml were cultured in replicate in 5-ml plastic Falcon 2063 tubes with specific test serum (30%) (normal, stimulatory, inhibitory or leukemic pretreatment sera) and Roswell Park Memorial Institute Medium 1640 (70%). The number of cells on Day 0 varied for each marrow because of the variable volumes of the bone marrow aspiration and the differing cell density of each individual marrow. However, the number of cells for each individual experiment was constant for the given concentrations on Day 0, which ranged from 2.09 to 3.25 × 10⁶ cells/ml. Cultures were incubated at 37° in a humidified atmosphere containing 5% CO₂. On Day 2 the supernatant was decanted and fresh media with serum at 30% were added to a total volume of 1 ml. Incubation was continued through Day 6. The sera were used in the following sequences (S, stimulatory; I, inhibitory; L, leukemic pretreatment): S → S, S → I, S → L, I → S, I → I, L → S, L → L.

[³H]TdR Incorporation Assay

This method has been described previously (1). Results are expressed as the percentage of cpm of [³H]TdR incorporated into the acid-insoluble precipitate from cells cultured in stimulatory, inhibitory, and leukemic pretreatment sera relative to cpm obtained from cells cultured in normal sera during the 1st 18 hr of culture.

Preparation of Microautoradiographs

[³H]TdR LI's were determined on cultures of 4 AML bone marrow cells. [³H]TdR, 0.1 μCi/ml, was added to cultures

initiated in stimulatory and in leukemic pretreatment sera on Day 0, to cultures in leukemic pretreatment sera on Day 1, and to cultures L → S and L → L on Day 2 and was incubated for 18 hr. Autoradiographs were prepared as previously described (1), and LI's were determined by the number of cells per 1000 counted that contained 5 or more grains overlying the nucleus. Background labeling was determined by the number of grains present in a cell-free area equivalent to the area of the myeloblast nucleus. However, >90% of all cells counted as having incorporated [³H]TdR label contained approximately 50 grains overlying the nucleus. Results are reported as the percentage of labeled myeloblasts. Our S.E. of this method was determined to be ±1%.

NCC and Granulocyte Morphology

NCC's were determined for replicate cultures of each serum type on Days 0, 2, 4, and 6. Because of the necessary variability in Day 0 counts, the results are expressed for the purpose of comparison as the percentage of Day 0 NCC. Viability of these replicate cultures was determined on the same day as WBC by trypan blue dye exclusion. The cells were then pelleted at 400 × g for 15 min, the supernatant was discarded, and 1 drop of 22% bovine serum albumin (pH 7.5) was added to each tube. Duplicate slides were prepared for each serum type, stained with buffered Wright's solution, and blinded. Differential counts of cells of the granulocytic series were obtained under ×1000 magnification for 200 cells/slide. Our S.E. of this method was determined to be ±3%.

RESULTS

[³H]TdR Incorporation and [³H]TdR LI

Stimulatory sera consistently increased [³H]TdR incorporation in both normal and leukemic cell types, whereas inhibitory and leukemic pretreatment sera caused similar suppression of [³H]TdR incorporation in both when compared with normal serum (Chart 1). The [³H]TdR incorporation by normal proliferative, AMMOL, and AML cells in stimulatory sera is significantly different from the incorporation in normal ($p < 0.01$), inhibitory ($p < 0.005$), and leukemic pretreatment sera ($p < 0.005$). The incorporation in inhibitory and leukemic pretreatment sera differs from normal sera ($p < 0.01$), but the incorporation in inhibitory sera does not differ from the incorporation in leukemic pretreatment sera ($p > 0.10$).

The [³H]TdR incorporation into an acid-insoluble precipitate and the [³H]TdR LI's of myeloblasts for the 4 AML bone marrows incubated in stimulatory or leukemic pretreatment sera on Day 0 and studied after 18 hr of culture correlated with a correlation coefficient of 0.87. LI increase was noted in those cells initially cultured in leukemic pretreatment sera and given stimulatory sera on Day 2 (L → S) ($p < 0.001$), whereas those cells maintained with fresh leukemic pretreatment sera on Day 2 demonstrated no increase (L → L, $p > 0.10$; Chart 2).

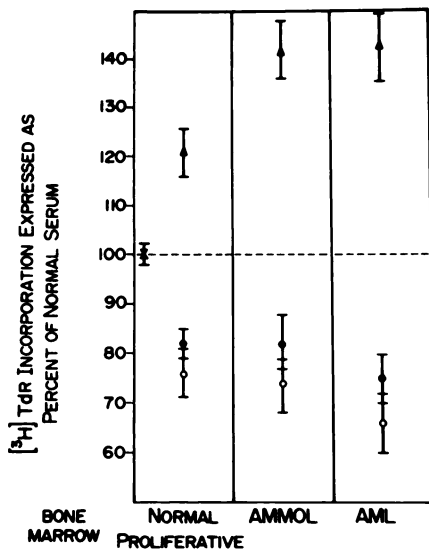


Chart 1. The effects of stimulatory (▲), inhibitory (●), and leukemic pretreatment (○) sera on $[^3\text{H}]\text{TdR}$ incorporation by the 3 types of bone marrow target cells (normal proliferative, 4 experiments; AMMOL, 3 experiments; and AML, 4 experiments) during the 1st 18 hr of culture. These data are expressed as the percentage of $[^3\text{H}]\text{TdR}$ incorporation relative to the incorporation by these cells in normal serum (x). Vertical bars, ± 1 S.E.

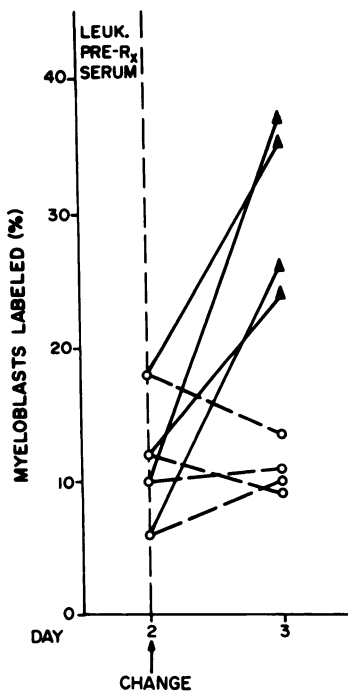


Chart 2. $[^3\text{H}]\text{TdR}$ LI's for the 4 AML bone marrows during the 1st 18 hr following serum and media changed on Day 2. Cultures were initiated in leukemic pretreatment serum (LEUK. PRE- R_x SERUM) on Day 0 and on Day 2 were given more leukemic pretreatment serum (L \rightarrow L, \bigcirc --- \bigcirc) or stimulatory serum (L \rightarrow S, \bigcirc — \blacktriangle).

Nucleated Cell Count

NCC paralleled the $[^3\text{H}]\text{TdR}$ incorporation of normal and leukemic cell types cultured in stimulatory, inhibitory, and leukemic pretreatment sera during the 1st 2 days (Chart 3). Stimulatory sera increased cell counts in all 3 bone marrow types during the 1st 2 days of culture ($p < 0.005$), and

addition of stimulatory sera to all culture types on Day 2 also resulted in increased cell counts [S \rightarrow S (\blacktriangle — \blacktriangle), $p < 0.01$; I \rightarrow S (\bullet — \blacktriangle), $p < 0.01$; L \rightarrow S (\bigcirc — \blacktriangle), $p < 0.005$]. Inhibitory and leukemic pretreatment sera acted initially in a cytostatic fashion on both normal and leukemic target cells and did not differ in their effects on normal proliferative, AMMOL, or AML cells ($p > 0.05$).

There was a decrease in NCC when inhibitory or leukemic pretreatment sera were added to all cultures initiated in stimulatory sera, with a greater effect evident in cultures of normal proliferative cells ($p < 0.01$ for normal cells, $p < 0.05$ for leukemic cells). Viability as determined by trypan blue dye exclusion was not reduced in either normal or leukemic cell cultures in any sera throughout the culture period.

Granulocytic Series Differentials

Cultures Initiated in Stimulatory Sera (S \rightarrow S, S \rightarrow I, S \rightarrow L). Stimulatory sera maintained the proliferative compartments (blasts, progranulocytes, myelocytes) of the normal proliferative marrow cultures. When more stimulatory sera were added on Day 2 to these maximally stimulated cells, there was further increase in proliferative forms by Day 4, with subsequent maturation as the nonproliferative compartment increased from 18% on Day 2 to 41% by Day 6 ($p < 0.01$; Chart 4).

When these stimulated cells were placed in an inhibitory milieu on Day 2 by addition of either inhibitory sera, which is the temporal sequence of serum activity observed *in vivo* following bone marrow ablation by drug, or by addition of leukemic pretreatment sera, there was a decrease in proliferative cells. This was associated with an observed increase

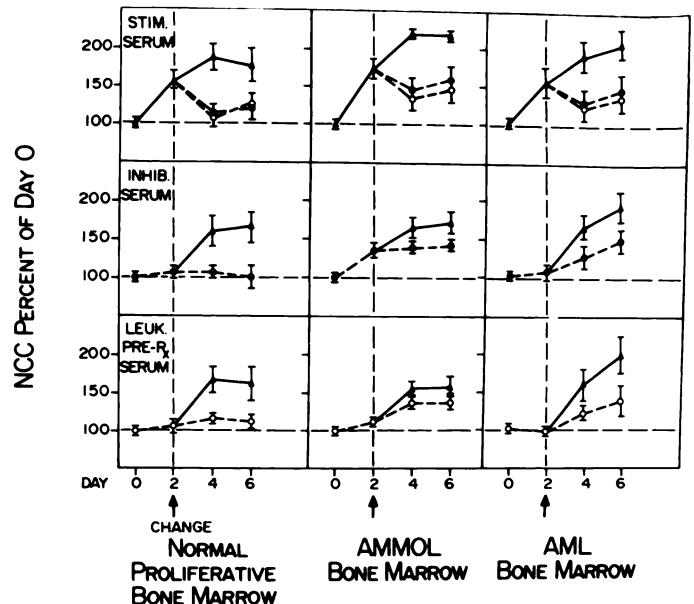


Chart 3. Nucleated cell count, expressed as the percentage of Day 0 NCC ± 1 S.E., during the 6 days of culture. Sera are depicted by the appropriate symbols [stimulatory serum (STIM. SERUM), \blacktriangle ; inhibitory serum (INHIB. SERUM), \bullet ; leukemic pretreatment serum (LEUK. PRE- R_x SERUM), \bigcirc ; S \rightarrow S, \blacktriangle — \blacktriangle ; S \rightarrow I, \blacktriangle --- \bullet ; S \rightarrow L, \blacktriangle --- \bigcirc , etc.]. The data are expressed as the percentage of Day 0 NCC. Averages for the 4 normal proliferative bone marrow, 3 AMMOL bone marrow, and 4 AML bone cell cultures are shown.

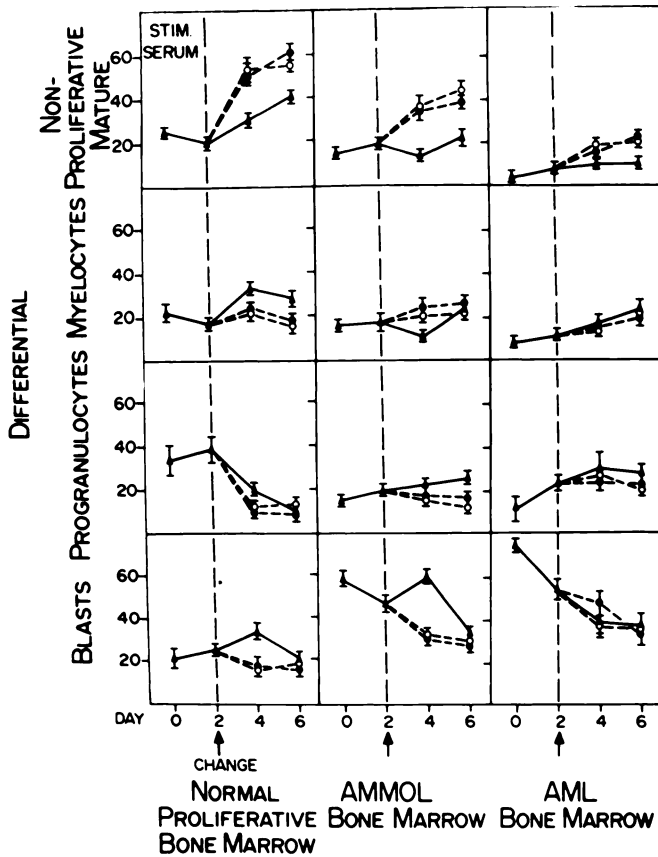


Chart 4. Granulocytic series differentials for all 3 bone marrow cell types \pm 1 S.E. in cultures initiated in stimulatory serum (STIM. SERUM) on Day 0 and fed with stimulatory (S \rightarrow S, \blacktriangle — \blacktriangle), inhibitory (S \rightarrow I, \blacktriangle — \bullet), or leukemic pretreatment (S \rightarrow L, \blacktriangle — \circ) serum on Day 2.

in mature cells from 18% on Day 2 to 52 to 60% on Days 4 to 6 ($p < 0.0005$).

The 2 leukemic cell populations exhibited somewhat different maturative capacities. Although AMMOL cells demonstrated a greater capacity for maturation than did AML cells, normal maturity was never attained. Myeloblasts of both leukemic cell types decreased during the 1st 2 days in stimulatory sera. AMMOL cells did not show significant changes in the other compartments, whereas the proportion of progranulocytes increased in AML cell cultures from 12% on Day 0 to 25% by Day 2 ($p < 0.01$). Addition of more stimulatory sera on Day 2 was associated with a subsequent rise in progranulocytes and myelocytes ($p < 0.05$) without an increase in the mature nonproliferative compartment by Day 6. When the stimulated AMMOL cells were cultured in either inhibitory or leukemic pretreatment sera on Day 2, the percentage of proliferative forms decreased and gradual persistent increase in mature forms (18% on Day 2 to 33 to 42% by Days 4 to 6; $p < 0.01$) was noted. However, when stimulated AML cells were exposed to this sera sequence, the rise in mature nonproliferative forms was smaller than the rise seen with similarly treated normal proliferative cells (9% on Day 2 to 20 to 22% by Days 4 to 6; $p < 0.05$).

Cultures Initiated in Inhibitory and Leukemic Pretreatment Sera (I \rightarrow S, I \rightarrow I, L \rightarrow S, L \rightarrow L). Inhibitory (Chart 5) and leukemic pretreatment sera (Chart 6) were similar in their effects. In the cultures of normal proliferative bone

marrow cells, suppression of the proliferative compartments (predominantly progranulocytes) was evident, with a concomitant rise in mature forms from 25% on Day 0 to 40 to 43% on Day 2 ($p < 0.005$). When more inhibitory or leukemic pretreatment sera were added on Day 2, a further small rise to 48% in the mature compartment was noted by Day 4. However, by Day 6, an increase in proliferative forms had occurred in both inhibitory and leukemic pretreatment sera, concomitant with a rise in NCC (Chart 3). With the addition of stimulatory sera to those inhibited cells on Day 2, there was a prominent rise in myeloblasts and progranulocytes by Day 4, with subsequent increase in the more mature compartments by Day 6.

Again, leukemic cells exhibited a decreased ability to mature in either inhibitory or leukemic pretreatment sera, with greater impairment evident in AML versus AMMOL cells. A decrease in myeloblasts was noted for both throughout the culture period, with some increase in the proliferative compartments when stimulatory sera were added on Day 2. Increase in the more mature compartments did not ensue. In AMMOL cell cultures, there was some increase in mature forms (14% on Day 0 to 23 to 26% on Day 2; $p < 0.025$), with gradual increase to 30% by Day 6 subsequent to addition of more inhibitory or leukemic pretreatment sera on Day 2. In AML cultures, however, there was no significant rise in mature forms in inhibitory sera throughout the culture period. In leukemic pretreatment sera, how-

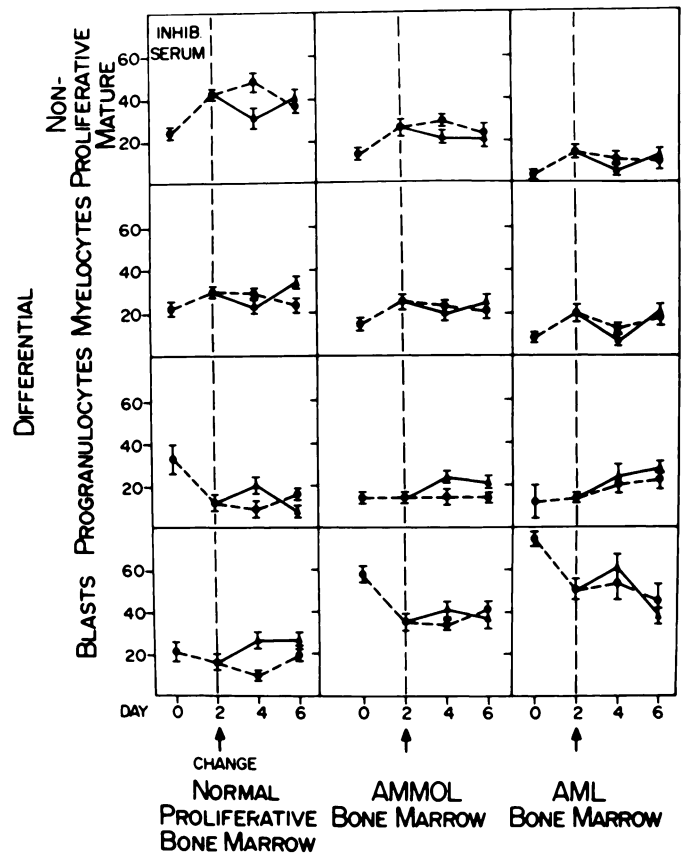


Chart 5. Granulocytic series differentials for all 3 bone marrow cell types \pm S.E. in cultures initiated in inhibitory serum (INHIB. SERUM) on Day 0 and fed with stimulatory (I \rightarrow S, \bullet — \blacktriangle) or inhibitory (I \rightarrow I, \bullet — \bullet) serum on Day 2.

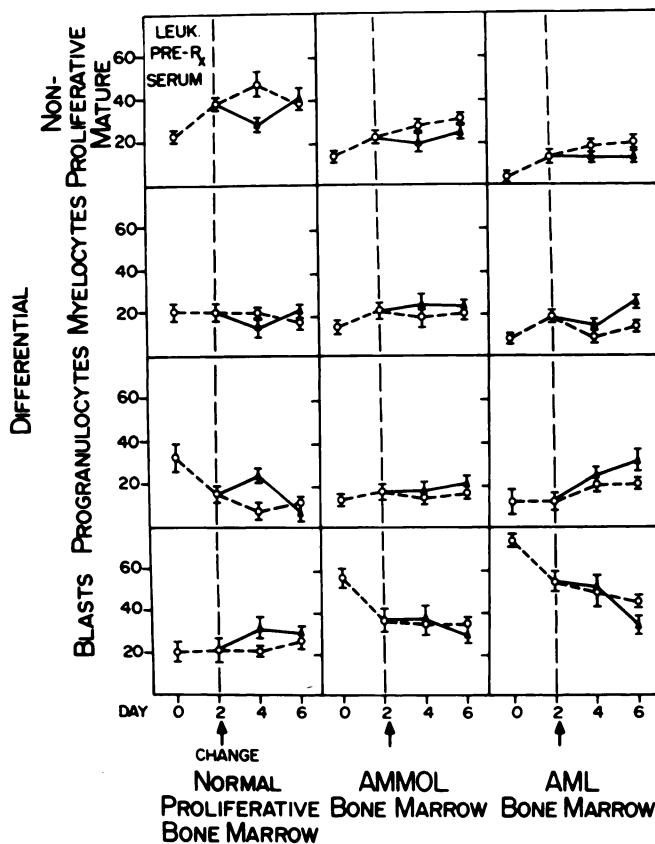


Chart 6. Granulocytic series differentials for all 3 bone marrow cell types ± 1 S.E. in cultures initiated in leukemic pretreatment serum (LEUK. PRE-R, SERUM) on Day 0 and fed with stimulatory (L \rightarrow S, \circ — Δ) or leukemic pretreatment (L \rightarrow L, \circ --- \circ) serum on Day 2. Results in all cultures initiated in leukemic pretreatment serum are qualitatively and quantitatively similar to results achieved in inhibitory serum (Chart 5) throughout the 6 days of culture.

ever, there was a rise in mature forms from 4% on Day 0 to 16% by Day 2 ($p < 0.05$) and 20% by Day 6 ($p < 0.025$).

The normal proliferative marrow is the closest morphological counterpart to AMMOL and AML that we can obtain. There were striking differences in observed differentials, particularly in the mature nonproliferative compartment, between normal cells and leukemic cells, even though cell proliferation as measured by $[^3\text{H}]\text{TdR}$ incorporation and NCC was similar.

DISCUSSION

From these *in vitro* studies, we have shown a direct relationship between serum regulatory factors and bone marrow cell proliferation. Stimulatory serum, obtained *in vivo* during the early phase of normal bone marrow recovery following drug-induced aplasia, effects proliferation of both normal and leukemic cells in culture. Inhibitory serum, obtained during the late phase of normal bone marrow recovery, suppresses proliferation of both normal and leukemic cells in culture. Furthermore, these studies demonstrate that leukemic pretreatment sera are similar to inhibitory sera in their retardation of proliferation of both normal and leukemic cells.

Correlation of the $[^3\text{H}]\text{TdR}$ incorporation assays with the LI's of AML bone marrow cells documents enhanced cellu-

lar proliferation of those leukemic cells exposed to stimulatory sera. Similarly, $[^3\text{H}]\text{TdR}$ incorporation and NCC's in normal marrow cell cultures paralleled those of leukemic cell cultures. Thus, proliferation of normal cells in stimulatory serum also occurred. That these cultures contained an increased proportion of proliferative forms relates either to an absolute increase or to senility and death of mature granulocytes. The observed increase in NCC supports the 1st hypothesis. It is possible that other nucleated cells, such as erythroid or lymphoid cells, could have accounted for the total increases in NCC. However, this is unlikely for the AML bone marrow cell cultures, which were $>95\%$ leukemic cells at the initiation of culture. Although the LI's confirm this increase in leukemic cell number for AML cell cultures, we did not exclude proliferation of other marrow elements in cultures of normal proliferative and AMMOL bone marrow cells.

Both inhibitory sera (inhibitory and leukemic pretreatment) acted in a cytostatic manner. Cells remained viable while in inhibitory sera yet rapidly proliferated when placed in stimulatory sera. However, there was a decrease in cell count when previously stimulated normal cells were exposed to these inhibitory sera. It is probable that the decrease in the number of previously stimulated cells was related to enhanced maturation and senescence of the proliferating normal cells. Leukemic cells, unable to attain comparable maturity, decreased minimally.

The results of these *in vitro* studies, in which sera containing peak physiological levels of induced humoral factors were used in sequence, are consistent with further postulates. Stimulatory sera effect recruitment into cell cycle of both normal and leukemic cells in culture and neither cause nor prevent cellular maturation. Leukemic pretreatment sera, commonly inhibitory to proliferation (10, 13, 18), are functionally similar to normal inhibitory sera, as both enhance maturation while suppressing proliferation of normal bone marrow granulocytes. This inhibitory effect of leukemic pretreatment sera, which may account for the low proliferative index of leukemia at clinical presentation (4, 14, 25), is not leukemogenic in this system. The impaired maturation of leukemic cells, which in some systems is not totally refractory to *in vitro* manipulation (6, 22, 23), probably resides within the malignant cells and seems a relative defect (8, 11, 13, 16, 19). Different types of myelocytic leukemia display a varied capacity for maturation, as AML cells remain more primitive than do AMMOL cells. However, although we might not detect monocytic differentiation in these short-term cultures, a definite aberration in granulocytic development was apparent in AMMOL.

Serum regulatory factors stimulate and inhibit normal and leukemic bone marrow cell proliferation *in vitro*. Any influence on the inherent capacity of the cell to mature seems indirect. The specific and consistent cellular defect displayed by leukemic cells in this system is that they do not mature under optimal sequence of serum regulatory substances.

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