Effect of Erythropoietin on RNA Synthesis by Normal and Leukemic Bone Marrow and Spleen Cell Suspensions In Vitro

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Erythropoietin (EPO) induced a 42% increase in ^{3}H-uridine incorporation into RNA after a 5-hr culture of normal bone marrow cell suspensions. Bone marrow cells obtained from rats 3–5 days after the initiation of a myelogenous leukemia exhibited a decreased responsivity to EPO. At this time incorporation of the isotope into RNA in the presence of EPO was approximately 50% of controls. Rats rendered leukemic 8–10 days prior to culture showed no bone marrow response to EPO even in those instances where leukemic cells comprised a relatively small percentage of the marrow compartment. EPO had little or no effect on RNA synthesis by spleen cells obtained from normal and leukemic rats. This was noted even in those leukemic spleens in which erythropoiesis was observed. The data suggest that the anemia associated with myelogenous leukemia may, in part, be due to a loss of EPO-responsive cells and/or a loss of sensitivity of these elements to normal humoral control.

ALTHOUGH THE OCCURRENCE of anemia associated with leukemias is well documented,[1] fundamental mechanisms whereby leukemogenesis interrupts erythropoiesis remain obscure. Observations of a marked reduction in the ability of rats implanted with a myelogenous leukemia to synthesize heme have prompted investigations of early steps in erythroid cell proliferation and development.[2,3] The earliest effect of erythropoietin (EPO) on macromolecular synthesis by normal rat bone marrow and mouse fetal liver cells in vitro is an increase in RNA synthesis.[4,5] The present study was designed to examine the influence of the leukemic state on RNA synthesis by hemic cell populations subjected to EPO. Our findings confirm previous observations that EPO stimulates RNA synthesis in normal rat bone marrow.[6] However, this stimulatory effect of the hormone is lost during the course of the leukemia. There is a reduction in the EPO response in the early stages of the leukemia followed by a complete absence of the stimulatory effect of the hormone in the intermediate and late stages of the disease. The presence of more mature hemoglobinized erythroid cells in the cultures suggested that RNA synthesis by these cells, as well as nonerythroid elements, is not affected by the hormone. Little or no detectable stimulation of RNA synthesis by EPO was noted in either normal or leukemic spleen cell cultures.

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MATERIALS AND METHODS

Animals

All animals used were male Long-Evans rats weighing between 180 and 200 g maintained on a diet of Purina Lab Chow and tap water ad libitum. Rats were rendered leukemic by the injection of 10×10^6 leukemic cells via the jugular vein, and assays were performed 3–10 days postinoculation. The maintenance of the leukemic colony, preparation of leukemic cell suspensions, and the time course of this myelogenous leukemia has been previously described.[7]

Cell Suspensions

Surgical equipment and glassware were dry-heat sterilized, media and sera were filtered through a 0.2 μ Nalgene Filter Unit (Nalge, Sybron Corp., Rochester, N.Y.); all procedures were performed under a vertical laminar flow station (Baker Co.).

Bone Marrow

Bone marrow cell suspensions were prepared by aseptically excising the femurs from each test animal. The femur head was removed and the marrow flushed through with 2.0 ml of incubation media using a 21-gauge needle and hypodermic syringe. The marrow was then passed through a 44.0 μ nylon mesh (Nytex, Tobler and Traber Co., Elmsford, N.Y.) secured in a Swinny Hypodermic Adapter (Millipore Filter Corp., Bedford, Mass.) attached to a 5.0-ml syringe. An aliquot of the resultant suspension was removed and the total number of nucleated cells determined. Cells were pipeted into tubes as described below; the remaining suspension was mildly centrifuged and the cell button smeared. Duplicate slides were prepared for treatment with May-Grunwald and benzidine-hematoxylin stains. Previous studies[7] have shown that the percentage of bone marrow leukemic myeloblasts is the most stable criterion for the sequential staging of the disease process. Each leukemic animal was used separately and the data correlated with the progress of the leukemia.

Spleen

Spleens were aseptically removed and placed in a sterile petri dish. The spleen was then bisected, each half held firmly in place with the aid of forceps, and 2.0 ml of incubation media repeatedly flushed through the organ until sufficient numbers of cells had been harvested. Samples were prepared for incubation and differential examination.

Chloroma

Subcutaneous tumors (chloromas) were aseptically excised from the donor animals. Pieces of tumor were placed in a sterile glass hand homogenizer with a Teflon pestle which had been previously ground down so as to fit very loosely. The incubation media were added and a cell suspension prepared by moving the pestle vertically several times. The suspension was filtered through sterile glass wool and an aliquot removed for counting and differential analyses. Appropriate cell numbers were pipeted into tubes for subsequent incubation and assay.

Incubation

The medium consisted of 40% freshly heat-inactivated (56°C, 30 min) fetal calf serum (BBL), 58% NCTC-109 (Microbiological Associates) and 2% of 7.5% $NaHCO_3$. Cells were cultured in Falcon plastic culture tubes (12 × 75 mm) at 37°C in a 5% CO_2, 95% air atmosphere. Each tube contained 350 μl of cell suspension (30×10^6 cells/ml). All samples were tested in triplicate. Erythropoietin (0.2 U/ml, 58.8 U/mg)* where used was added to culture tubes at the beginning of the incubation period.

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*The erythropoietin was collected and concentrated by the Department of Physiology, University of Northeast, Corrientes, Argentina, and further processed and assayed by the Hematology Research Laboratories, Children's Hospital of Los Angeles, under Grant HE 10880 (National Heart and Lung Institute).

RNA Extraction

Each sample was pulsed with (5-^{3}H) uridine (specific activity 25 Ci/mmole, New England Nuclear) at a concentration of 4μCi/ml for 20 min. At the end of this time incubations were terminated by placing the tubes in an ice-water bath; the incubation mixtures were then centrifuged in a clinical centrifuge (2000 rpm) at 4°C for 3 min. The overlying medium was removed and the cell pellet washed once with medium, centrifuged, resuspended, and finally precipitated with 0.1 ml of 0.1 *M* $Na_2P_2O_7$ and 1.5 ml 7.5% TCA containing 0.001 *M* ATP. This suspension was then filtered on glass-fiber filters (Whatman GF/C) saturated with 0.01 *M* ATP, washed three times with 10 ml of ice-cold 5% TCA, and once with 5 ml of 95% ethanol. The filters were oven dried (30 min at 60°C) and counted in a toluene-based scintillation fluid (Liquifluor, New England Nuclear).

In order to determine if ^{3}H-uridine was incorporated into RNA, we used the methods of Monroe and Flick[8] and Schneider[9] as modified by Hausen and co-workers.[10] TCA-insoluble material was dissolved in 0.3 *N* KOH. The material (RNA) was hydrolyzed by incubating overnight at 37°C. A predetermined amount of 1.2 *N* HCl was added to neutralize the KOH, and the mixture was adjusted to 0.25 *N* $HClO_4$with 70% $HClO_4$. The precipitate was removed by centrifugation and the total RNA calculated from absorbance of the supernatant at 260 nm. Colorimetric determinations were performed by orcinol analysis, and an aliquot was counted in aquasol. The precipitate was hydrolyzed in 1 ml 4% TCA for 15 min at 90°C and counted in aquasol. Radioactivity was measured in a Packard β-liquid Scintillation Counter.

RESULTS

The effect of EPO on ^{3}H-uridine incorporation into RNA by normal bone marrow cells in vitro is shown in Fig. 1. The data indicated that a 5-hr incubation period was optimum to study the EPO effect. In 12 separate experiments assayed at this time, EPO stimulated isotope incorporation into RNA by a mean of 42.4% (range, 26.7%–75.6%). Tritiated uridine incorporation into spleen cell suspensions obtained from normal rats was only slightly stimulated at 3 hr by EPO (Table 1). It is possible to equate ^{3}H-uridine incorporation into trichloroacetic acid-precipitable counts with RNA synthesis if there is no appreciable change in the intracellular pool of RNA precursors. We determined relative pool size by incubating control and hormone-treated cultures using three different levels of the isotope. The results, given in Table 2, are consistent with a constant intracellular pool.[11]

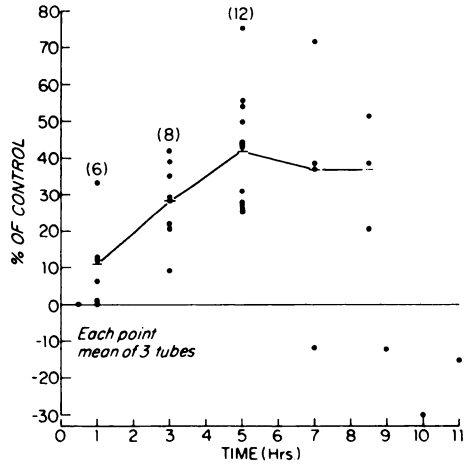

Fig. 1. The effect of EPO on ^{3}H-uridine incorporation into RNA by normal rat bone marrow cells in vitro. (The curve represents the mean; numbers in parentheses indicate the number of experiments.)

Table 1. Effect of EPO on ^{3}H-uridine Incorporation Into RNA of Hemic Cell Suspensions Prepared From Normal Rats

Tissue	Expts.	Per Cent Benzidine-Positive Cells	3-hr Incubation CPM −EPO	3-hr Incubation CPM +EPO	3-hr Incubation Per Cent Change	No. Expts.	5-hr Incubation CPM −EPO	5-hr Incubation CPM +EPO	5-hr Incubation Per Cent Change
Bone Marrow	8	24.8 ±3.0*	1490 ±190	1890 ±230	+28.3	12	1420 ±150	2030 ±240	+42.4
Spleen	4	3.4 ±0.8	1410 ±70	1480 ±180	+4.9	4	1470 ±270	1460 ±280	−0.7

*Mean ± SEM.

The rate of RNA synthesis by pure populations of leukemic myeloblasts prepared from solid tumors was not influenced by EPO (Table 3). Table 4 presents data for bone marrow and spleen suspensions from 11 animals sacrificed 8–10 days after the intravenous administration of leukemic cells. EPO failed to stimulate RNA synthesis by bone marrow cells even at a time when leukemic cells comprised a relatively small percentage of the marrow compartment. Slides prepared from aliquots of bone marrow harvested from animals L1–L5 indicated the presence of normal numbers of benzidine-positive cells. In animals L6–L11, benzidine-positive cells, though present, were appreciably reduced. Isotope incorporation into RNA by spleen cell suspensions obtained from animals L1–L11 was in all but one animal unaffected by exogenous EPO despite the routine observation of hemoglobinized cells in these samples.

In order to determine whether the time after leukemic cell administration was of significance, we examined the bone marrow and spleen cells of animals rendered leukemic 3–5 days prior to assay. The data obtained from seven rats are presented in Table 5. Bone marrow myeloblasts in these rats ranged from 0.1% to 26%. At this time (3–5 days post-leukemic-cell administration) EPO stimulated RNA synthesis by bone marrow cells of some of the animals studied (L12–L14). In bone marrow cells of rats (L15–L18), however, there is a marked reduction in the ability of exogenous EPO to augment ^{3}H-uridine incorporation into RNA. Spleen cells in this series were also refractory to exogenous EPO. Benzidine-positive cells were present in both bone marrow and spleen of animals L13–L18. Bone marrow and spleen slides prepared from L12 appeared normal; few nucleated erythroid cells were present in the spleen. The mean per cent stimulation of isotope incorporation by EPO in L12 (42.9%) was compa-

Table 2. Effect of ^{3}H-uridine Specific Activity on Incorporation Into RNA by Bone Marrow Cells in Culture

Specific Activity of ^{3}H-uridine (μCi/30 × 10^6 cells)	3-hr Incubation CPM −EPO	3-hr Incubation CPM +EPO	Per Cent Change
15	4290 ±210	5350 ±410	+26.3
8	2700 ±290	3390 ±230	+25.6
4	1480 ±200	1870 ± 30	+24.5

Table 3. Effect of EPO on ^{3}H-uridine Incorporation Into RNA of Pure Populations of Leukemic Cells Prepared From 10-day Subcutaneous Tumors

	3-hr Incubation			5-hr Incubation		
Tumor	CPM −EPO	CPM +EPO	Per Cent Change	CPM −EPO	CPM +EPO	Per Cent Change
1	3670	3390	− 8.9	1210	1090	− 9.4
2	3560	3180	−10.8	2810	2760	− 1.8
3	1530	1500	− 1.7	2660	2840	+ 6.8
4	3000	2000	−33.3	3120	1950	−37.3
5	5130	4760	− 7.3	4770	4880	+ 2.3

Table 4. Effect of EPO on ^{3}H-uridine Incorporation Into RNA of Bone Marrow and Spleen Cell Suspensions Harvested From Rats 8–10 Days After the Onset of the Myelogenous Leukemia

	Bone Marrow					Spleen				
Animal	Per Cent Benzidine-Positive Cells	Per Cent Blast	CPM −EPO	CPM +EPO	Per Cent Change	Per Cent Benzidine-Positive Cells	Per Cent Blast	CPM −EPO	CPM +EPO	Per Cent Change
L1	24.3	0.1*	1360	2150	+58.1	1.3	0	640	760	+19.4
L2	22.3	3.0	2540	1930	−24.1	2.0	1.5	1090	920	−15.8
L3	20.4	5.4	2570	2180	−15.3	—	—	—	—	—
L4	22.3	7.0	1890	1890	0	—	—	—	—	—
L5	19.8	29.0	4580	4810	+ 5.0	8.0	3.2	570	410	−28.5
L6	6.8	44.0	2460	2700	+ 9.7	5.2	73.5	4740	3780	−20.2
L7	9.2	54.0	2700	2620	− 2.9	8.3	1.9	320	350	+ 8.7
L8	3.4	58.5	7760	7450	− 3.9	—	—	—	—	—
L9	5.1	63.0	8960	7780	−13.2	19.1	2.5	770	380	−50.2
L10	< 1.0	97.0	5660	5660	0	14.7	63.0	5730	5270	− 8.0
L11	< 1.0	97.0	5960	6710	+12.6	6.1	65.3	5520	4640	−16.1

*Denotes per cent leukemic myeloblast in cell suspension.

Table 5. Effect of EPO on ^{3}H-uridine Incorporation Into RNA of Bone Marrow and Spleen Cell Suspensions Harvested From Rats 3–5 Days After the Onset of the Myelogenous Leukemia

	Bone Marrow					Spleen				
Animal	Per Cent Benzidine-Positive Cells	Per Cent Blast	CPM −EPO	CPM +EPO	Per Cent Change	Per Cent Benzidine-Positive Cells	Per Cent Blast	CPM −EPO	CPM +EPO	Per Cent Change
L12	22.1	0.1*	1400	2040	+42.9	1.2	0.5	1000	710	−10.9
L13	24.6	2.1	1500	1800	+20.5	6.4	0.7	940	610	−35.0
L14	23.3	2.8	1990	2670	+33.8	7.7	1.5	810	890	+ 9.2
L15	14.1	3.6	4350	4710	+17.2	7.2	0.6	1040	990	− 5.3
L16	19.2	4.4	3000	3530	+17.8	6.4	0.8	1480	1470	0
L17	14.6	15.4	2890	3710	+16.1	11.4	33.0	2260	2800	+ 5.4
L18	19.6	26.0	3300	3420	+ 3.8	4.1	69.0	3410	3850	+12.9

*Denotes per cent leukemic myeloblast in cell suspension.

rable to normal values. These data suggest that the loss of ability by bone marrow elements to respond to EPO depends both on the presence of leukemic cells in the marrow compartment as well as the length of time after seeding of these cells. Although there is a diminished response to EPO 3–5 days after the initiation of the leukemia, the abolition of the response occurs only after further in vivo exposure. The observation that RNA synthesis in both marrow and spleen cultures from leukemic rats was unaffected by EPO despite the presence of maturing hemoglobinized erythroid cells in culture suggests that RNA synthesis by more mature erythroid elements as well as the nonerythroid populations in the hematopoietic compartment is unaffected by the hormone.

DISCUSSION

We have shown that the stimulatory effect of EPO on RNA synthesis by bone marrow of leukemic rats is reduced as early as 3–5 days after the onset of the leukemia and completely disappears 8–10 days after the transplant of leukemic cells into the host. The failure of EPO to stimulate RNA synthesis in 8–10-day leukemic bone marrow was evidenced even when leukemic myeloblasts comprised only a small fraction of the total marrow population. As the leukemia progressed, the number of benezidine-positive elements in the femoral marrow decreased. We believe that the remaining hemoglobinized erythroid cells are the progeny of precursors which passed through the erythropoietin-dependent stage prior to the "inhibitory" influence of the leukemic state. In the animal, these committed erythroid elements continue to mature until their release into the circulation. Using mouse fetal liver cells, Djaldetti et al.[5] have demonstrated that EPO had no effect on the rate of RNA synthesis by nonerythroid cells or by more mature hemoglobinized erythroblasts in culture. The diminished response to EPO reported in our study indicates that during the course of the leukemia there is either a marked reduction in the numbers of EPO-sensitive cells and/or that these developing precursors, though present, lose their sensitivity to the hormone and fail to differentiate into the mature cell line, thus accounting for the reduction of the numbers of observed hemoglobinized cells in later stages of the leukemias.

The data show an increase in control RNA synthesis in both the marrow and spleen samples in which there are appreciable numbers of leukemic cells present (Tables 4 and 5). We believe that this elevation in ^{3}H-uridine incorporation into RNA is due to the RNA-synthetic activity of leukemic myeloblasts in the cultures. An alternate hypothesis is that under the influence of leukemic cells there is maximal RNA synthesis by erythroid precursors, independent of the normal regulator (EPO). Militating against this interpretation is the decrease in the numbers of erythroid colonies formed by leukemic marrow in clot cultures,[12] loss of heme-synthetic capacity of erythroid cells,[3] and the anemia seen in late stages of the disease.[7] Mirand has observed "unregulated erythropoiesis" in animals subjected to a variant of the Friend virus.[13] However, these organisms manifest a polycythemia.

The size of spleen and bone marrow CFU-S compartments of mice has been shown to be altered after infection with Friend and Rauscher leukemia virus, suggesting that the pluripotential colony-forming unit may be a target cell for these viruses.[14] Thomson and Axelrad[15] suggest that two types of CFU-S may

exist after Friend leukemia virus infection, one which can be destroyed with the appropriate antiserum and another which is resistant. Although the successful isolation of virus particles from the Shay chloroleukemic cell has not been reported, "C"-type particles have been observed both in intercellular spaces[16] and budding from leukemic myeloblasts.[17] Whether these particles are present in sufficient numbers to affect the host hematopoietic tissue is not known. Neither is there information regarding changes in the pluripotent stem cell pool during the course of this transplanted myelogenous leukemia. We have, however, observed no decrease in soft-gel granulocyte/macrophage colonies (CFU-C) in the early and intermediate stages of the leukemia, suggesting that these committed cells are present in normal numbers.[18] Studies in progress in our laboratory indicate that when leukemic bone marrow suspensions are put into plasma clot culture the numbers of erythroid colonies which develop are markedly reduced, indicating a decline in the numbers of available erythropoietin-responsive cells (CFU-E). The latter have been suggested as the cells which give rise to erythroid colonies.[19] We have also observed that EPO, when added to these plasma clot cultures, fails to stimulate the development of erythroid colonies.[12] The pattern that emerges is one in which the leukemic state reduced both the numbers and response of erythroid precursors to normal regulatory control.

Ernst et al.[20] have found that basophilic erythroblasts obtained from patients suffering from acute and chronic myelogenous leukemia incorporated only small quantities of exogenous thymidine into DNA, giving the impression that no DNA synthesis was taking place. After inhibition of de novo synthesis of nucleoside triphosphates, these erythroid cells obtained from patients with AML and CML showed the capacity for precursor incorporation. These workers suggest that a biochemical abnormality results, where high intranuclear triphosphate pools may inhibit nucleoside kinases and hence utilization as well as dilution of the labeled precursor. This defect was interpreted as a biochemical expression of "leukemicness" of maturing erythroid cells. Our studies on a transplanted leukemia indicate that the erythroid compartment expresses its "leukemicness" early in erythroid development, either through a decline in erythropoietin-sensitive erythroid elements or a loss of erythropoietin sensitivity by precursor cells with ultimate failure in maturation.

During the course of this myelogenous leukemia, progressive alterations in bone marrow architecture, including destruction of marrow sinuses, have been demonstrated[17,21] and may contribute to the decline in the erythroid precursor pool. Trentin and co-workers[22] have found that the microenvironment in which hematopoietic cell lines mature exerts a marked effect on the differentiation of erythroid and granulocytic cells. McCuskey et al.[23] have suggested that stem cells committed to the erythroid line will complete their development only in a microenvironment that is highly vascularized, has a high rate of blood flow, and contains neutral mucopolysaccharides; this would constitute the elements of the microenvironment of erythropoiesis (HIM-E). Biochemical changes which accompany reduced and altered microvasculature may in part affect this transplanted myelogenous leukemia. If this is the case, the effect on the erythroid compartment is of such a nature as to interfere with the normal development of EPO-responsive cells, which ultimately results in the development of anemia.

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