Leukemic Host Influence on Normal Erythrocytic and Granulocytic Colony Formation in in Vivo Plasma Clot Diffusion Chamber Cultures

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ABSTRACT

The effect of a leukemic environment on normal erythroid and granulocytic colony formation was examined in in vivo plasma clot diffusion chamber cultures implanted into Shay chloroleukemic rat hosts at varying stages of the disease. Normal bone marrow cells isolated in plasma clot diffusion chamber cultures in leukemic hosts displayed significant differences in the pattern of normal bone marrow colony growth. Granulocyte colony-forming units were significantly inhibited by leukemic hosts throughout the course of the disease. The size of developing colonies was reduced to under 100 cells; however, maturation within these clusters appeared unaffected. Erythroid colonies showed a slight inhibition during the early stages of the leukemia, a significant stimulation of 100 to 350% in the midleukemic period, and a significant inhibition of 50 to 65% during the terminal stages of the disease. Burst formation was also inhibited in the late leukemic stages. The transient increase in erythroid colony-forming units on Days 7 and 8 of the leukemia was concomitant with the onset of the anemia associated with the disease. Since the normal bone marrow cells were compartmentalized within the plasma clot diffusion chamber cultures, the suppression of erythroid and granulocytic colony development appears to be directly due to the release of diffusible inhibitory substances from the leukemic animal.

INTRODUCTION

The decrease in normal granulocyte and erythroid elements in the bone marrow and peripheral blood of humans and experimental animals with AML is associated with disturbances in in vitro colony formation (4, 7, 8). Both the reduction in in vitro colony development and/or the formation of abortive clusters have been observed. Whether this suppression is due to a decline in pluripotential or committed stem cells as a result of a direct leukemic cell-cell interaction or the loss of their functional capacities under the influence of leukemic cell-inhibitory substances is as yet not resolved. While evidence supporting both possibilities has been advanced (16, 19, 26), the underlying mechanisms in leukemogenic suppression of granulopoiesis and erythropoiesis in situ are not well known.

In an effort to understand the factors and events leading to the suppression of normal hematopoiesis in acute leukemia, the effect of a leukemic environment on normal erythroid and granulocyte colony formation was directly evaluated in PCDC's implanted into Shay chloroleukemic host rats. In a previous study, the PCDC technique was introduced as a reliable in vivo culture system for the assessment of erythroid and granulocyte progenitors within the physiological environment of the host animal (23). By seeding normal bone marrow into PCDC's and implanting them into differentially perturbed hosts, the factors influencing alterations in colony development and the functional state of erythroid and granulocyte progenitors can be evaluated. In applying this concept and technique to the study of normal hematopoiesis in acute leukemia, experiments were designed to examine the development of normal bone marrow progenitors in PCDC's implanted into leukemic hosts at various stages of the disease. By this approach, alterations in erythroid and/or granulocyte colony formation could be monitored throughout the course of the disease. Since the normal bone marrow cells are isolated within the PCDC's, leukemic influence on normal hematopoiesis via cell-cell interaction or the release of diffusible leukemic cell products can be more clearly distinguished.

MATERIALS AND METHODS

All animals used were male Long-Evans rats weighing 180 to 250 g, maintained on a diet of Purina laboratory chow and tap water ad libitum. Untreated rats were the source of normal bone marrow cells for culture in diffusion chambers. Chambers were implanted into normal untreated hosts and rats having an AML (Shay chloroleukemia). Experimental animals were made leukemic 0, 4, and 6 days prior to chamber implantation by i.v. injection of a suspension of leukemic cells at a concentration of 5 x 10⁶ cells/100 g body weight. Hematocrits and peripheral leukocyte determinations were made on the free-flowing tail blood of all chamber recipients prior to anesthesia. Bone marrow smears were stained with benzidine and Wright's-Giemsa stain and assessed for the percentage of bone marrow myeloblasts and percentage of benzidine-positive cells.

Cell Suspensions and Medium. All surgical equipment and glassware were dry heat sterilized. Medium consisting of 20% heat-inactivated fetal calf serum in McCoy's Medium 5A was passed through a 0.2-µm Nalge filter unit to prevent bacterial contamination. Preparation of cell suspensions and surgical procedures were performed under a vertical laminar air flow unit. Both femurs of normal donor rats were aseptically removed, and the bone marrow was flushed out with media, using a hypodermic syringe. A cell suspension was prepared by

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4 The abbreviations used are: AML, acute myelogenous leukemia; PCDC, plasma clot diffusion chamber culture; CFU-C, granulocyte colony-forming unit; BFU-E, erythroid colony-forming unit; BFU-E, erythroid burst-forming unit.

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passage through a 44.0-μm nylon mesh. An aliquot was removed for total and differential analysis. Cells were diluted to the appropriate concentration and stored on ice. Preparation of leukemic cell suspensions and the induction of the Shay chloroleukemia has been described previously (5, 6).

(PCDC). The preparation, harvest, and fixation of PCDC’s has been previously described in detail (23). Briefly, diffusion chambers constructed with 0.22-μm GS type Millipore filters were seeded with 5 × 10⁵ normal bone marrow cells in 120 μl of medium, followed by the addition of 20 μl of citrated bovine plasma. Each chamber was immediately stoppered, heat sealed, shaken vigorously to ensure a uniform distribution of cells within the chamber, and set aside for 3 to 5 min until the clot was formed. Completed chambers were transferred into ice-cold medium where they remained until implantation.

To assess the development of normal erythroid and granulocytic stem cells within the leukemic environment, chambers were implanted into leukemic animals at various stages of the disease. In Experiment I, early stage leukemic hosts received PCDC’s several hr after tumor cell inoculation. Middle- and late-stage leukemic recipients were implanted with PCDC’s 4 and 6 days after disease induction in Experiments II and III, respectively. Each animal within the group received 4 chambers in the peritoneal cavity. Chambers implanted into normal untreated rats served as controls.

**Scoring of Colonies.** Clot preparations are evaluated at × 400. Colonies are examined according to type, number of cells per colony, and the degree of cell maturation within each colony. Granulocyte colonies are scored when consisting of 20 cells or more. Erythroid colonies are scored as benzidine-positive units of 4 to 7 cells or greater. Two classes of erythroid colonies are counted, depending upon the spatial orientation of the erythroid colonies within the clot matrix. Erythroid colonies appearing on Day 2 of culture are randomly dispersed throughout the clot matrix, while those occurring on Day 7 are grouped into larger aggregates called bursts. Each burst is counted as a single large colony consisting of 6 to 8 smaller erythroid units. Both the individual colonies comprising the burst as well as the burst itself (larger aggregate) are evaluated separately.

**RESULTS**

The development of PCDC granulocyte colonies within the leukemic environment of rat hosts at various stages of the leukemia is represented in Chart 1. Colony growth was assessed in 250 PCDC’s implanted into 82 leukemic rats. Control values for CFU-C, CFU-E, and BFU-E obtained from PCDC’s implanted into normal hosts were similar to those previously reported (23). In Experiments I and II, significant inhibition (p < 0.003; p < 0.005) of granulocyte colony formation ranging between 10 and 60% with a mean of 36% of control values, was observed over the entire course of the disease. In terms of cell number, the size of these developing granulocytic colonies was dramatically reduced to under 100 cells as compared with colonies consisting of several hundred cells observed in normal host rats. Maturation of cells within the colony was unaffected with the appearance of normal metamyelocyte-mature neutrophil colonies. Granulocyte colony formation in 5 of 7 rats sacrificed showed a mild stimulation (p < 0.005) on Day 10 of the leukemia in Experiment III, with colonies appearing normal in size and maturation. Colony formation in the remaining 2 animals showed an inhibition of 24%. It is possible that the fluctuating data in this group of terminal-stage rats may result from mildly increased levels of colony-stimulating activity in response to leukemia-induced neutropenia. It should also be noted that the data for this group are incomplete since 10 to 15 rats died of the disease prior to chamber harvest and evaluation.

Erythroid colony formation (Chart 2) was mildly depressed (p < 0.03) during the early stages of the disease (Experiment I: hematocrit range, 47 to 32%), significantly stimulated (p < 0.05; p < 0.0005) to between 100 and 350% of control on Days 7 and 8 after leukemia induction (Experiment II: hematocrit range, 47 to 26%; and Experiment III: hematocrit range, 43 to 26%), and significantly inhibited (p < 0.01; p < 0.0003) to between 50 and 65% of controls in the terminal stages of the disease (Experiment II). As determined by the hematocrit, the transient increase in CFU-E on Days 7 and 8 after leukemia induction occurred with the onset of the disease-associated anemia. Burst formation was also shown to be inhibited in the middle and late leukemic stages (Experiments I and II). The numbers of individual colonies comprising the burst as well as the number of cells within each colony was noticeably reduced.

**Chart 1.** Granulocyte colony formation of normal bone marrow cells in PCDC’s implanted into 82 leukemic hosts on Day 0 (Experiment I), Day 4 (Experiment II), and Day 6 (Experiment III) of the leukemia. Each point represents mean granulocyte colonies as a percentage of control values from 2 to 4 chambers harvested from a single leukemic host. Bars, mean values for the percentage of control values of all chambers harvested for each experimental set on a selected day of culture during the course of the leukemia.
DISCUSSION

In vitro cultures of bone marrow from humans and animals with AML are predominantly characterized by the lack of colony development or the formation of large numbers of small abortive clusters composed of poorly differentiated cells (4, 8, 17, 18). The suppressive effect of leukemic cells on normal CFU-C proliferation and differentiation is believed to be due to the release of inhibitory substances from leukemic cells (1, 19). In the present study, abnormal colony formation in in vivo PCDC’s implanted into Shay chloroleukemic rats is consistent with disturbances in colony formation observed in vitro. Both the reduction in the total number of CFU-C’s and the development of abortive colonies were observed. Since the normal bone marrow cells are compartmentalized within the chamber, the suppression of normal CFU-C development can be directly attributed to the release of inhibitory diffusible substances from leukemic hosts and thus is not the result of a dilutional or crowding-out effect by an expanding leukemic cell population within the marrow. The reduction in CFU-C’s in the early stages of the leukemia (<10% medullary myeloblasts) tends to support this conclusion and is in agreement with observations of reduced colony formation in preleukemic states (4, 22). While the presently observed results may be due to diminished or altered formation of diffusible colony-stimulating activity, direct inhibition of CFU-C’s in vitro by leukemic cell extracts (1, 20, 21), tumor-conditioned media and serum from leukemic rats (8), strongly supports inhibition of PCDC colonies via leukemic cell inhibitor substances as a primary mode of stem cell inhibition.

In human AML, clusters are believed to be derived from leukemic elements (17, 18). However, the origin of tight clusters observed in Shay chloroleukemic rats is uncertain. The present study suggests a normal cell origin. Clusters developing in isolated PCDC’s are derived from the normal bone marrow cells seeded into them. The formation of abortive colonies in vitro and in PCDC’s may be linked to an inhibitory effect of leukemic diffusible substances on normal cells in the later stages of their differentiation.

In recent years, the work of Rytomaa (19) and others (26) have brought into focus the role of granulocytic chalone in the negative feedback control of normal granulopoiesis and in the pathogenesis of AML. In the Shay chloroleukemic rat, the excessive loss of chalone from leukemic cells results in an abnormal elevation in the endogenous serum levels of inhibitor (20, 21). The progressive increase in the percentage of leukemic myeloblasts in the bone marrow during the course of the disease would, therefore, lead to an increasing disturbance in the normally balanced relationship between stimulatory and inhibitory regulators of granulopoiesis. This would result in the suppression of normal CFU-C development as observed in vitro (8) and presently in PCDC’s implanted into leukemic rat hosts. The 36% reduction in CFU-C development observed in PCDC’s is in close agreement with and tends to elucidate the 33% decline in total cellularity in diffusion chamber suspension cultures harvested from Shay chloroleukemic rats (25). In chamber-bearing animals given injections with multiple doses of chalone, a 37% reduction in chamber cellularity has been noted (14). This reduction is correlated with a decrease in the number of proliferating CFU-C’s per chamber, as determined by recloning harvested cells into secondary in vitro agar cultures. While this suggests a direct effect of chalone inhibitor on the CFU-C’s, the present reduction of normal PCDC colony formation in leukemic rats provides more direct evidence for this control.

The development of anemia in human (11), mouse (13), and rat (7, 10) leukemia is believed to be an erythropoietic lesion in the stem cell compartment. In the Shay chloroleukemic rat, the decrease in the pattern of medullary erythropoiesis is related to the reduction in the numbers and/or the functional capacity of the committed CFU-E’s as reflected in the reduction in erythrocytic colonies formed in vitro plasma clot cultures (7). A similar reduction in erythroid colony formation is observed in PCDC’s implanted into leukemic rats. Both CFU-E’s and BFU-E’s were depressed in early- as well as late-stage leukemic hosts (Experiments I and II). The early suppression of erythroid colony formation in compartmentalized PCDC’s supports similar observations in vitro (7) and suggests that suppression, in situ, prior to heavy leukemic cell infiltration in the bone marrow, may be due to the elaboration of diffusible inhibitory substances in leukemic hosts. This possibility has been demonstrated by the reduction of in vitro CFU-E’s by media conditioned by leukemic tumor cells. Whether this inhibition is the result of substances specific for erythroid progenitors is presently under study.

\[1 \text{ E. S. Handler, unpublished data.} \]
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The release of diffusible inhibitory substances from leukemic hosts can help to explain the observed early shutdown of erythropoiesis (5–7, 9) and provide an understanding of the mechanisms involved in the in situ suppression of erythroid progenitor development and its loss of responsiveness to erythropoietin stimulation. The early inhibition observed in PCDC erythroid colony formation is reversed on Days 7 and 8 after leukemia induction (Experiments II and III), leading to a 100 to 350% increase in colony growth. This significant increase occurs at a time when the disease-associated anemia is readily observed (hematocrit, 27%) and high circulating levels of erythropoietin are present (3). Therefore, under the influence of a leukemic inhibitor, erythroid progenitors in PCDC’s are presumably suppressed, remain dormant, and do not respond to erythropoietin until the endogenous levels are high enough to override the effect of the inhibitor. The presence of erythropoietin-responsive erythroid progenitors in PCDC’s may support the view that, during the early and middle stages of the disease, medullary erythroid progenitors, in situ, do not decline in absolute numbers but become refractory to circulating levels of anemia-induced erythropoietin (12, 24, 27). The results, therefore, suggest that, in situ, the ability of erythropoietin to stimulate the differentiation of potentially active erythroid progenitors is disturbed. It is possible that during the course of the leukemia, the destruction of the proper erythroid microenvironment (2, 15) may result in the inability of erythropoietin to reach potentially responsive erythroid stem cells. During the late stages of the leukemia PCDC, colony formation is again inhibited. At this stage, the animal becomes highly debilitated, suffering from severe hepatomegaly, splenomegaly, marrow hypoplasia, and the eventual metastasis of leukemic cells into the central nervous system.

Our findings suggest that, during the course of this experimental leukemia, diffusible leukemic cell products effectively suppress normal hematopoiesis at a time prior to extensive leukemic cell infiltration into the bone marrow (cell-cell interaction) and continue to influence normal bone marrow cell proliferation throughout the course of the disease. Suppression is seen at the level of the committed stem cell. The leukemic cell population thus gains a proliferative advantage that leads to the eventual takeover of the normal hematopoietic compartment.

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