

THE LEUKEMIC CELL IN VIVO AND IN VITRO

Frederick Stohlman, Jr., M. D.*

The Department of Medicine, St. Elizabeth's Hospital
and
Tufts Medical School, Boston, Massachusetts

Before considering the problem of leukemia it seems appropriate to briefly review the normal regulation of hemopoiesis. I shall confine my remarks to normal myelopoiesis and those leukemias associated with disorders of myelopoiesis i. e. acute myelocytic, progranulocytic and undifferentiated stem cell leukemias as well as chronic myelocytic leukemias.

A schematic outline of normal myelopoiesis (1) is shown in figure 1. It is to be noted that the myeloid system shares a common precursor cell with the erythroid and megakaryocytic series. This common precursor cell is referred to as the pluripotent hemopoietic stem cell. Intermediate between the pluripotent stem cell and those recognizable myeloid elements, which may be identified under the light microscope, is a compartment which is committed to myelopoiesis. Similar compartments exist for the erythroid and megakaryocytic series. There is evidence, which has recently been reviewed (1), that suggests that monocytes and myeloid elements derive from a common committed precursor cell in which case the committed myeloid stem cell compartment can not be considered truly unipotent. Further the myeloblast and progranulocyte differentiates along one of three lines the neutrophil, eosinophil or the basophil. We do not know at what stage this commitment is made i. e. committed stem cell, myeloblast or progranulocyte, nor what is the decision making mechanism.

In animals there is a technique to evaluate the pluripotent stem cell compartment (2) but in man it cannot be assayed and inferences must be drawn from animal experimentation. Briefly the assay for the pluripotent stem cell or CFU consists of transplanting marrow or spleen from the animals in question into heavily irradiated syngeneic mice. The animals are sacrificed after 8–10 days by which time discrete colonies are formed in the spleen. These colonies arise from a single cell and morphologically may be erythroid, myeloid, megakaryocytic, differentiated or

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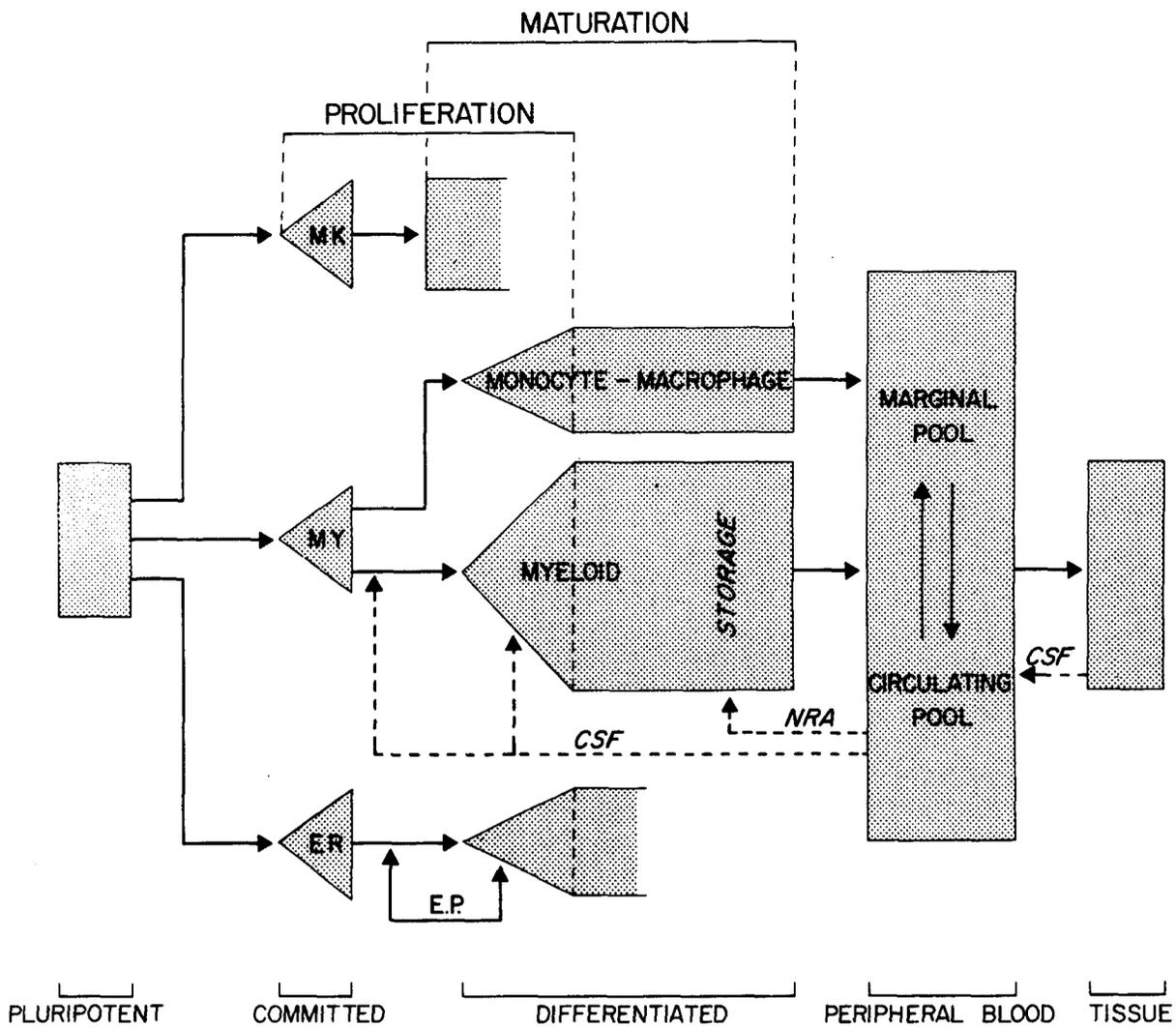


Fig. 1: Schematic representation of hemopoiesis: MK-Megakaryocytes; MY-Myeloid; ER-Erythroid; EP-Erythropoietin; CSF-Colony Stimulating Factor; NRA-Neutrophil Releasing Activity; solid line (EP) indicates established in vivo activity; dashed line (CSF) indicates putative activity. (From Progress in Hematology).

mixed. When a colony is dissected out and transplanted into a secondary recipient, the colonies formed from this inoculum will have the same spectrum of morphologic types as seen in the primary transplant, indicating the pluripotent characteristics of the cells giving rise to colonies as well as the capacity for self renewal.

The committed myeloid stem cell (CFC) may be evaluated in vitro by culturing bone marrow in soft agar (3, 4). The growth of cells from murine marrow is dependent upon the presence of a glycoprotein-colony stimulating factor. In man, however, autonomous growth is observed when the cell concentration in the dish is sufficiently high. The latter presumably reflects the presence in human marrow of a cell capable of producing the colony stimulating factor. This cell is most likely the monocyte (5, 6).

The hormone erythropoietin is responsible for the differentiation of erythroid committed cells into pronormoblasts and prolonged administration produces a sustained erythrocytosis. It is tempting to suggest that colony stimulating factor

serves a similar function for myelopoiesis but as yet firm evidence in the intact animal to establish a physiologic role of CSF has not been forthcoming. There are, however, indirect data from which it has been inferred that CSF differentiates the myeloid committed stem cells into myeloblasts in the intact animal (1).

After differentiation, the myeloid cells undergo a series of divisions while at the same time maturing. The capacity for further DNA synthesis ceases at the myelocytic stage but there is further maturation of the granulocytic elements within the bone marrow prior to release into the peripheral blood. Thus the bone marrow contains a large storage pool of nonproliferating granulocytes, metamyelocytes, bands and mature polymorphonuclear cells. It has been shown experimentally that certain experimental manipulations (7, 8), e. g. the administration of endotoxin, effect the release of a protein, which is capable of releasing granulocytes from the storage compartment into the peripheral blood; this plasma factor has been referred to as neutrophil releasing activity (1).

The mature granulocytes have a relatively short intravascular sojourn, the half-time being of the order of 6.7 hours (9). During this time the granulocytes are distributed between the central circulating blood and the margins of capillaries (9). Thus there is a marginal and circulating pool of granulocytes, which are normally in equilibrium. Granulocytosis may result from a shift of cells from the marginal to the circulating pool, as is seen after epinephrine.

There are several lines of evidence to suggest that myelopoiesis normally is governed by a feedback loop from the peripheral blood which is regulated by a function of the circulating neutrophils. A feedback loop requires a time delay between measurement of a particular function in the peripheral blood and correction by changing the production rate. This time delay would include an interval for the production of a regulatory hormone, its effect on the committed stem cell and the time required for maturation of the differentiated myeloblast and its progeny. From this consideration one would expect oscillations of the peripheral neutrophil count and further that the period of these oscillations would be twice the time delay for differentiation, maturation and release. Normally oscillations in the peripheral granulocyte values are not seen. This is thought to reflect the dampening effect of the intramedullary storage pool (10). If so, then removal of the storage compartment should lead to detectable oscillations in the peripheral granulocyte count. Morley and I (11) observed that when animals were given cyclophosphamide, thus reducing the stem cell compartment and in consequence the storage compartment, a cyclical neutropenia developed and the period of the oscillations was twice the projected time delay for bone marrow transit.

Further evidence for feedback control of granulopoiesis was provided by studies in which one hind leg of an animal was shielded while the remainder of the body was irradiated (12, 13). The shielded limb permitted us to measure the myeloid response to neutropenia. Different levels of neutropenia were produced by varying the dose of irradiation. As may be seen in figures 2 and 3 the concentration of both the committed myeloid compartment, as assessed by the CFC technique, and the differentiated proliferating myeloid cells (myeloblasts and promyelocytes) varied as a log-linear function of the peripheral neutrophil concentration. These data therefore provide firm evidence that myelopoiesis is regulated by a feedback loop from the peripheral blood.

It seems most likely that the mechanism regulating myelopoiesis is not the neutrophil concentration but rather a function thereof. We have observed, as neutropenia developed, an increase in colony stimulating factor (14) and a decrease in lipoprotein inhibitor of in vitro colony formation in irradiated animals (15). The time course suggested that the increased generation of CSF might be due to bacteremia and endotoxemia. This notion was supported by the observation that germfree animals did not develop colony stimulating factor after irradiation as did conventional animals (16). When treated with endotoxin, however, germfree animals did produce CSF (15). This led to the concept that endotoxemia was important in CSF generation and in further experiments in normal animals a dose response relationship was observed between the dose of endotoxin and the amount of colony stimulating factor generated (17). It is attractive to suggest therefore that the regulation of myelopoiesis in a significant measure is related to intermittent bacteremia or endotoxemia and perhaps also the breakdown products of normal tissues which result in the release of pyrogens or endotoxin. Thus in the face of local infection or tissue necrosis one would

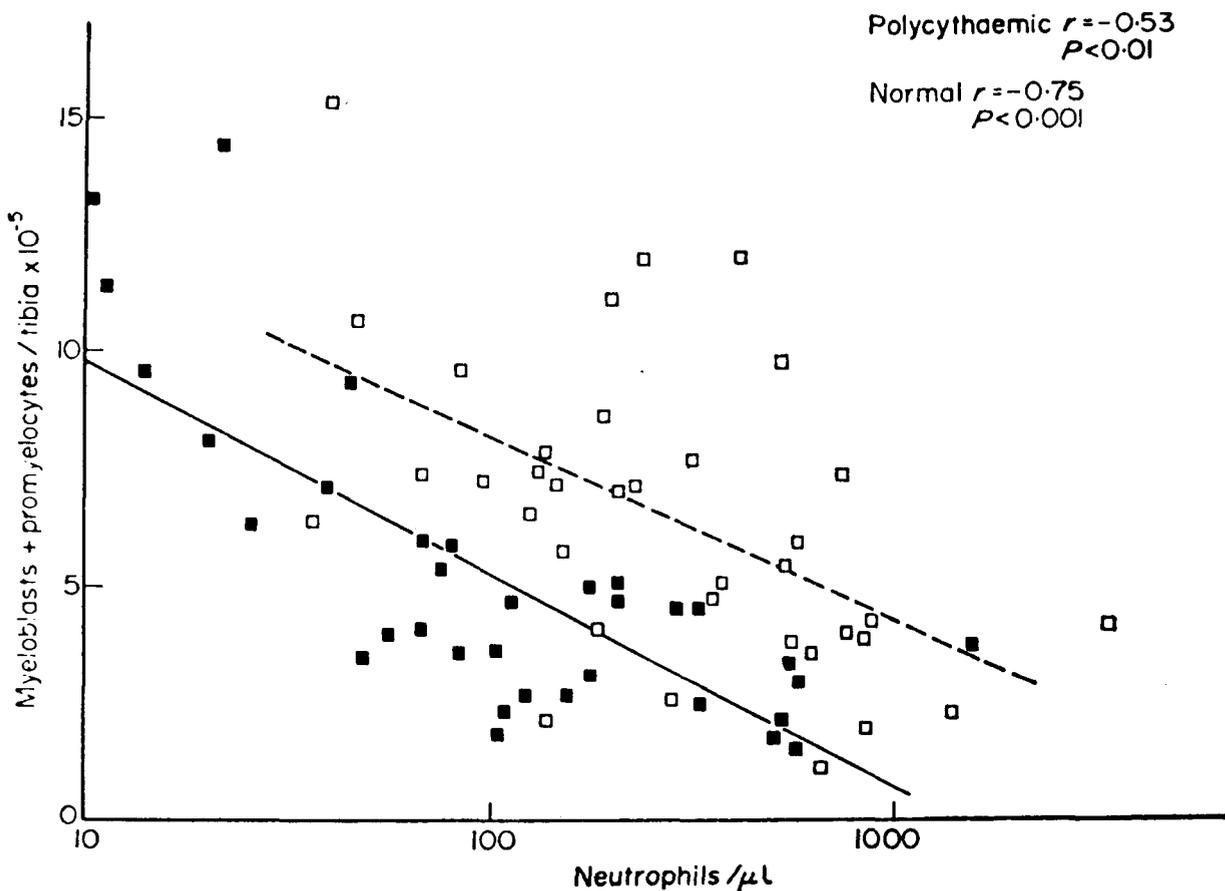


Fig. 2: Effect of polycythaemia on the size of the myeloblast-promyelocyte compartment on day 4 after various doses of irradiation. Each point shows the myeloblast-promyelocyte compartment compared with the neutrophil count from a polycythaemic (□) or normal (■) mouse. The regression lines were calculated by the method of least squares. In each case the size of the myeloblast-promyelocyte compartment is significantly ($P > 0.01$) related to the logarithm of the neutrophil count but the regression line for the polycythaemic animals lies significantly ($P > 0.01$) above that for the normal animals. (From Morley et al. 1970, reprinted from Br. J. Haematol. (24)).

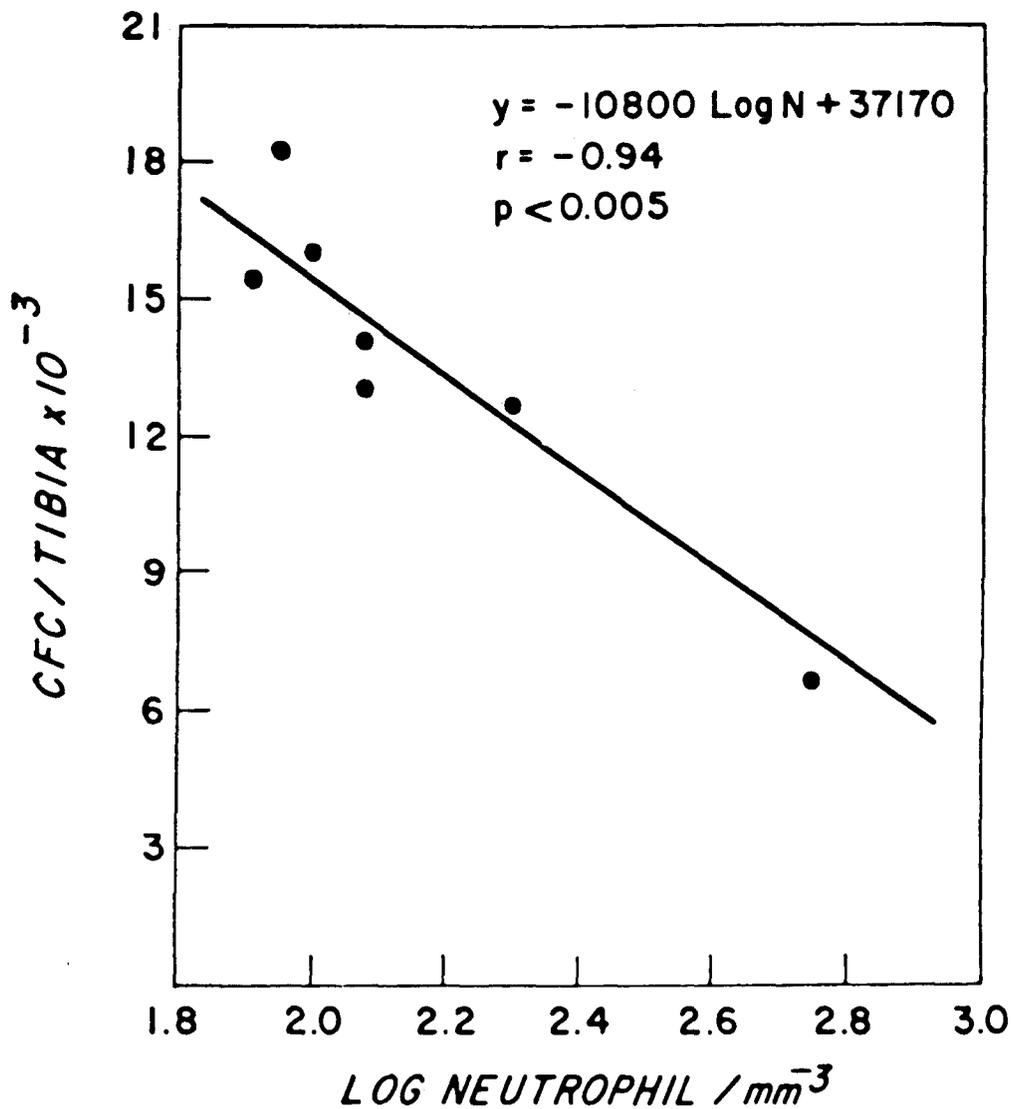


Fig. 3: The relationship between the size of the CFC compartment and the blood neutrophil count in ILS mice for days 2–8 after irradiation. CFC are the average of mean values for composite results of at least three experiments per point. Correlation coefficient r and the regression line are calculated from these values and are significant when $P > 0.005$. (From Rickard et al. 1971. reprinted from Blood (13).)

anticipate increased CSF production which would produce the granulocytosis, which is seen under such circumstances. Neutropenia of course is associated with infection and increased CSF generation has been reported (18).

This hypothesis is appealing but there are still a number of questions to be resolved, principally the problem of tolerance. It is well known that an animal repeatedly injected with endotoxin will become tolerant to its pyrogenic effects and this has been observed in respect to colony stimulating factor (15). Moreover, there appears to be cross-tolerance between various endotoxins.

A major problem in assessing the granulopoietic response to a variety of stimuli has been the storage pool. This has made it difficult to separate granulocytosis due to a

true increase in proliferation from release of granulocytes from the storage pool. Further, the possibility exists that the depletion of the storage pool triggers an intramedullary feedback loop which may affect myelopoiesis. The use of the millipore diffusion chamber has been introduced to circumvent this problem (19, 20). Normal bone marrow is implanted into these chambers which are then placed intraperitoneally into host animals. Manipulation of the hosts prior to implantation of the chambers provides an opportunity for the study of growth regulation within the *in vivo* culture system. Using this technique Tyler and his associate (21) have demonstrated that the proliferation of pluripotent stem cells as well as the growth of myeloid elements is enhanced in neutropenic animals. The best explanation for these results is that a humoral factor is produced in the neutropenic host which diffuses into the chambers. The nature of this humoral factor and its relation to the colony stimulating factor have not been established.

From the above we may conclude that myelopoiesis is governed by a feedback regulatory mechanism from the peripheral blood and tissues. There is indirect evidence to suggest that this regulation may be accomplished by the glycoprotein, colony stimulating factor, which is responsible for the differentiation of myeloid elements in the soft agar culture system. There is, however, no direct *in vivo* evidence that CSF plays this central role. Finally the mechanisms governing the production of CSF have not been completely resolved. It is clear that many tissues have the capacity to produce this protein and that endotoxin plays an important role in its generation. Further studies, however, will be necessary before final definitive conclusions can be drawn about its physiologic role in the regulation of myelopoiesis.

The Leukemic Cell

The two principal types of myelocytic leukemia, are the acute leukemias, including acute myelocytic, progranulocytic and myelomonocytic leukemia, and chronic myelocytic leukemia. Patients with chronic myelocytic leukemia after a variable period of time may enter what is termed a blast cell crisis which in all respects appears to be similar to acute myelocytic leukemia. This is most likely a reflection of the natural history of the disease although the role of chemotherapeutic agents and irradiation in effecting the transformation from CML to blast cell crisis has not been fully evaluated.

It is clear from cytogenetic studies that chronic myelocytic leukemia is basically a disease of the pluripotent stem cell compartment. The vast majority of patients have an abnormal chromosome, the Philadelphia chromosome (PH₁) which is seen in erythroid and megakaryocytic cells as well as in the myelocytic cells. Differentiation in many respects is normal and growth by and large is confined to the bone marrow and spleen, which might be considered a normal hemopoietic organ. The lack of the enzyme alkaline phosphatase in the mature granulocytes of CML indicates that differentiation is not entirely normal. Further, the intravascular life of the granulocyte in CML is prolonged. Immature cells, myelocytes, promyelocytes and myeloblasts are released into the peripheral blood suggesting some change in the transit pattern from bone marrow to peripheral blood. It has been suggested that the release of granulocytes is normally regulated by membrane properties (22). If so, then the premature release of immature cells suggest a change in the membrane and is further

evidence of abnormal differentiation. Although there are these demonstrable abnormalities of the granulocytes, it is clear that in some measure normal regulatory mechanism persists. Oscillation of the peripheral white count and also the platelet count has been documented in a number of patients with CML (23), indicating the existence of a feedback mechanism. In most instances, however, the period of oscillation is longer than normal.

The prolonged life span of the granulocytes together with the retention of a measure of feedback control suggests that during its initial phase chronic myelocytic leukemia in part is an accumulative disease. Undoubtedly there is additional input from the stem cell compartment, because arithmetically the prolonged intravascular life span could not account for the level of granulocytosis seen in some patients nor for the oscillation seen in those where oscillatory phenomenon have been documented.

The acute myelocytic leukemias may show varying patterns. In some, examination of the bone marrow reveals only abnormal myeloid elements; in others erythropoiesis and megakaryocytopoiesis may be present. Even during the preleukemic phase there may be identifiable morphologic abnormalities in megakaryocytes and erythroid elements. When cytogenetic abnormalities exist, these may be present in the megakaryocytes and erythroid elements as well as the myeloid. These observations suggest that acute myeloblastic leukemia at least in some instances originates from the stem cell. It seems likely to me that this is true for all patients with acute myeloblastic leukemias. Of paramount importance in considering the pathophysiology of acute myelocytic leukemia is the fact that complete remissions can be achieved with chemotherapy and normal hemopoiesis re-established. Thus, normal pluri-potent stem cells must be present in the bone marrow together with the leukemic stem cells. This suggests the existence of clones of pluripotent cells some of which are leukemic the other normal. Thus, if leukemia is of viral origin the abnormal information transmitted by the virus is present in some but not all pluripotent stem cell lines. One might suggest as a corollary that in some instances the viral infection of a stem cell will result in a lethal transformation and death of what might otherwise have become a leukemic clone.

In others non-lethal information develops and an abnormal line of differentiation results producing leukemia. In still others the viral infection may not be manifest or affects DNA in such a way that normal myelopoiesis is unaffected. The alternative explanation of course would be that some stem cells become infected with virus but others do not.

Since the introduction of the soft agar technique for the culture of myeloid precursors there has been considerable interest in a number of laboratories as to the role of the myeloid committed stem cell and the differentiating colony stimulating factor in leukemic patients. It has been reported that normal differentiation may be achieved when bone marrow from a leukemic patient is cultured in vitro. In a rather large survey Moore (24) found that the marrow from about 60 % of patients with acute leukemia developed small clusters in culture rather than normal colonies, 20 % developed colonies and 20 % failed to grow. The central issue is whether in cultures of bone marrow from leukemic patients normal colonies develop from a normal pluripotent stem cell or under these cultural conditions are normal myeloid elements differentiated from a leukemic stem cell line? Moore and Metcalf (25) have reported

that cytogenetic markers present in leukemic cells were found in all metaphases of cultured leukemic marrow. Unfortunately pooled rather than single colonies were examined. These data indicate that leukemic cells are proliferating in culture but do not provide the final answer that normal granulocytes develop in culture from leukemic progenitors. To be candid there is relatively little data published on the morphologic aspects of the differentiated colonies from culture of leukemic cells and in a recent conference the photomicrographs which were shown of leukemic cultures did not persuade me that anyone had succeeded in differentiating leukemic cells into normal myeloid elements. It has also been suggested by Mintz and Sachs (26) that an inhibitor may be involved in the failure of leukemic myeloblast to differentiate.

Clearly, further information is needed on the characteristics of leukemic cells in the soft agar system. Are normal stem cells differentiating? Are leukemic cells capable of normal differentiation and the production of normal differentiated polymorphonuclear cells? What, if any, is the role of colony stimulating factor in the failure of differentiation? What, if any, is the role of inhibitors in this failure of differentiation?

The concept that myeloid leukemia represents merely a failure of differentiation from the myeloblast stage due to an impaired interaction between colony stimulating factor and the target cells seems to oversimplify the issue. This would be somewhat analogous to pernicious anemia in which the morphologic abnormalities may be as striking as those seen in acute leukemia. This disease has been shown to be the result of abnormal differentiation due to a deficiency of B₁₂. Administration of the vitamin immediately corrects the abnormal proliferation. It seems to me that those who consider acute leukemia in this light overlook one major and important characteristic of the leukemic cell. In pernicious anemia the abnormal cellular proliferation is confined to the normal areas of hemopoiesis — the bone marrow. In acute myelocytic leukemia as in other neoplastic diseases proliferation occurs not only in the site of normal cell production but elsewhere in the body. Thus in acute myelocytic leukemia leukemic proliferation may be seen virtually anywhere, the skin, the heart, the liver, brain, etc. Thus the normal mechanisms which restrict cell production to a specific anatomic area are no longer operative. It seems most likely that the normal mechanism controlling the site of cell production is an interaction between the cells and their immediate environment or microenvironment. Thus in acute myelocytic leukemia the growth pattern reflects not only a failure of differentiation but other cellular abnormalities, most likely in the membrane structure, which permit metastatic growth. In this respect acute myeloblastic leukemia appears to be more than a simple failure of differentiation of the myeloblast. For this reason it seems unlikely that the resolution of acute leukemia will result from efforts directed solely at differentiation of myeloblasts.

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