NORMAL GRANULOPOIESIS AND ITS ALTERATIONS IN MURINE MYELOGENOUS LEUKEMIA[1]

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ABSTRACT

This paper presents a review of the current knowledge of the process of granulopoiesis, and the kinetics and control of the process. The author makes a case for the understanding of normal developmental mechanisms and their control as a basis to the study of malignancy.

Stimulatory factors regulating the processes of proliferation, maturation, and release of mature granulocytes from the marrow are described. Inhibitory factors, such as chalone and the intermediate-molecular-weight protein "X" are also related to the overall control mechanism.

Application of control factors and antiserum to the stimulatory factors in the therapy in murine myelogenous leukemia has produced interesting results. An antiserum to the proliferation-stimulating factor, given prophylactically, produces prolonged survival and maintains "normal" hematologic values. Both the macroglobulin maturation factor and antiserum to it are effective in therapeutic regimens, prolonging survival and "normal" hematologic values. Finally, the inhibitor "X" has produced extended survival in therapeutic use.

These results suggest that the leukemic state is due to an alteration in the normal control mechanism and not to a permanent alteration of the cells. If these findings are born out in further studies, myelogenous leukemia stands in direct contrast to most forms of neoplasia, where cells are permanently transformed. Other laboratories have already suggested that this is the case for human myelogenous leukemia.

INTRODUCTION

The exciting evidences presented for the implication of a C-type RNA virus in murine and human leukemia have produced striking changes in the broad pattern of leukemia research. The past several years, have seen the discovery of reverse transcriptases (RNA-dependent DNA polymerases), and of the presence of these enzymes in human leukemic cells and in virus-like particles isolated from human milk (Temin and Mizutani, 1970; Baltimore, 1970; Gallo, *et al.*, 1970; Schlom, *et al.*, 1971). Virus-specific antigens have been described which can be used to identify suspected leukemia viruses and which lead to a dream of "cancer vaccines." Only a short time ago, this degree of progress was undreamed of.

Every burst of optimism, however, must be tempered by the cold facts of a realistic world. The discovery of specific inhibitors for the viral reverse transcriptases was tempered by the reality that this was not a "magic bullet" that would strike down cancer viruses wherever they occurred, but rather *might* be effective at certain points in the course of the disease. The discovery of virus antigens and the possibility of a vaccine has been tempered by the "oncogene hypothesis," or the alternative "provirus concept," and its supporting evidence, which suggest that the viral nucleic acid is transcribed to DNA and the viral genome becomes incorporated into the chromosomes of the host cell, thus being protected against attack.

Unless a "magic bullet" drug or a cancer vaccine is produced in the immediate future, a more basic approach must be considered. Because leukemia in particular and neoplasia in general involve alterations in proliferation and differentiation of cells, a full and clear understanding of normal developmental processes and the control of these events is basic to a "cure" or to prevention of the disease. Secondly, if the changes in the developmental process resulting in myelogenous

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leukemia are discovered, a series of opportunities for therapeutic management of the disease are presented. Thirdly, if a clear picture of the disease process in leukemia is obtained, much light will be shed on other forms of cancer, whether they are varying manifestations of a single disease or discrete entities. All are related in their alterations of the growth and developmental process, and in the fact that increased knowledge of development must hasten the time when errors in the process may be corrected or avoided.

For these reasons, our laboratory has begun to apply the limited but increasing knowledge of factors controlling the development of granulocytic leukocyte (granulopoiesis) to the study of possible alterations in this control mechanism in murine myelogenous leukemia. Whether a particular malignancy is virus-induced or a result of genetic or environmental changes, we feel that it is now possible to learn what changes are produced in the developmental process by these carcinogens.

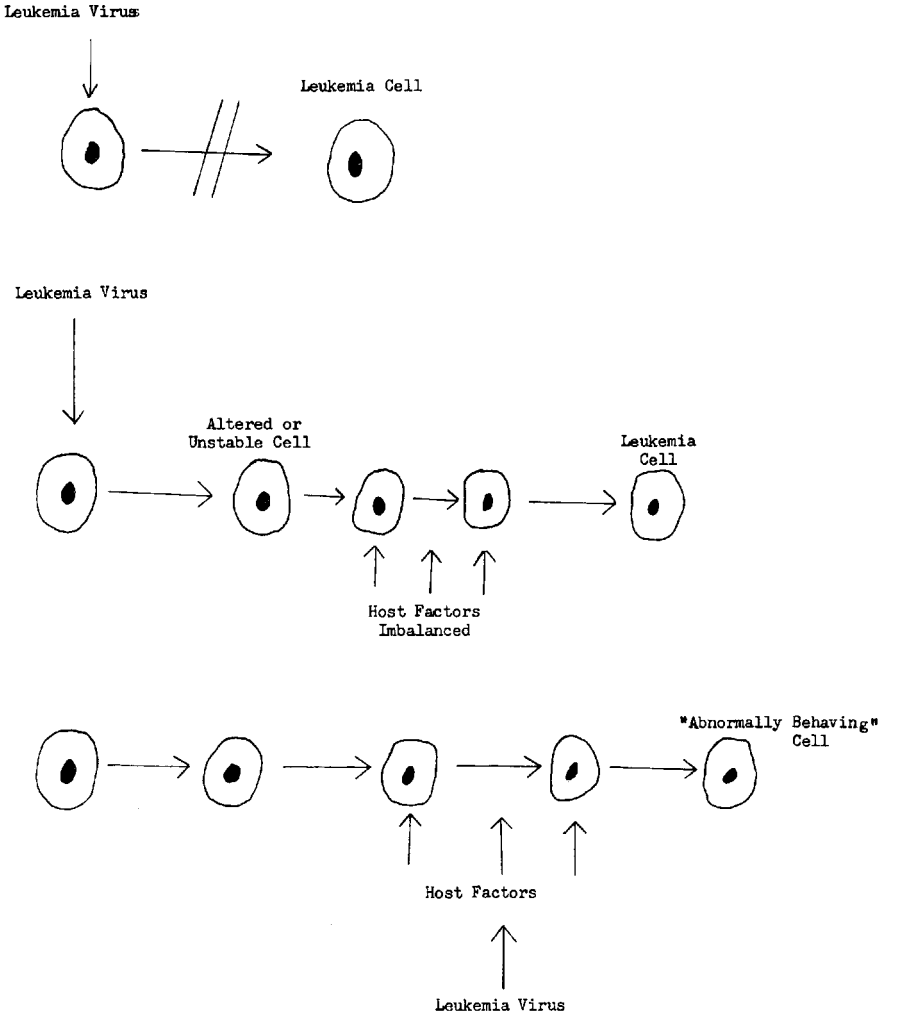

FIGURE 1. Top—Leukemia virus permanently altering host cells. Middle—Leukemia virus renders cell unstable and more sensitive to imbalances of control factors. Bottom —Leukemia virus does not affect cell but causes imbalance of control factors.

In studying an altered developmental process, two types of changes must be considered. First, the undifferentiated (primitive, embryonic, stem) cell which gives rise to the mature fully differentiated granulocyte may itself be altered (fig. 1A). Thus, whether the control mechanism functions properly or not, the cell can not follow the normal differentiation process and reach maturity. Secondly, the undifferentiated cell may be completely normal, but be unable to reach maturity because of damaged or changed control process (fig. 1B and 1C). Without the proper external stimuli, the cell would be partially or totally unable to become a fully differentiated granulocyte.

Before discussing the hypothesis that myelogenous leukemis is a manifestation of an altered control mechanism for the process of a granulocyte development, a clear understanding of normal granulopoiesis must be present. This is especially true because the literature in the field is cluttered with several sets of synonomous nomenclature. This already-complicated procedure has been rendered thoroughly befuddled by an unstandardized vocabulary and irreproducible experimentation. Therefore, it seems useful to set down a brief synthesis of what is known and what remains unknown about the process of granulocyte development, and its kinetics and regulation.

PATHWAYS OF MYELOPOIESIS

In the early days of experimental hematology, there existed as many theories for the developmental origin of the different blood cells as there were laboratories. One extreme was the polyphyletic school, with a belief that erythrocytes, granulocytes, lymphocytes, monocytes, and perhaps megakaryocytes came from individual ancestral, or "stem," cells, with no relation between groups except in the embryo (Doan, 1932; Sabin, *et al.*, 1936). The other was the monophyletic belief that a common "stem" cell was the forerunner of all the different mature blood

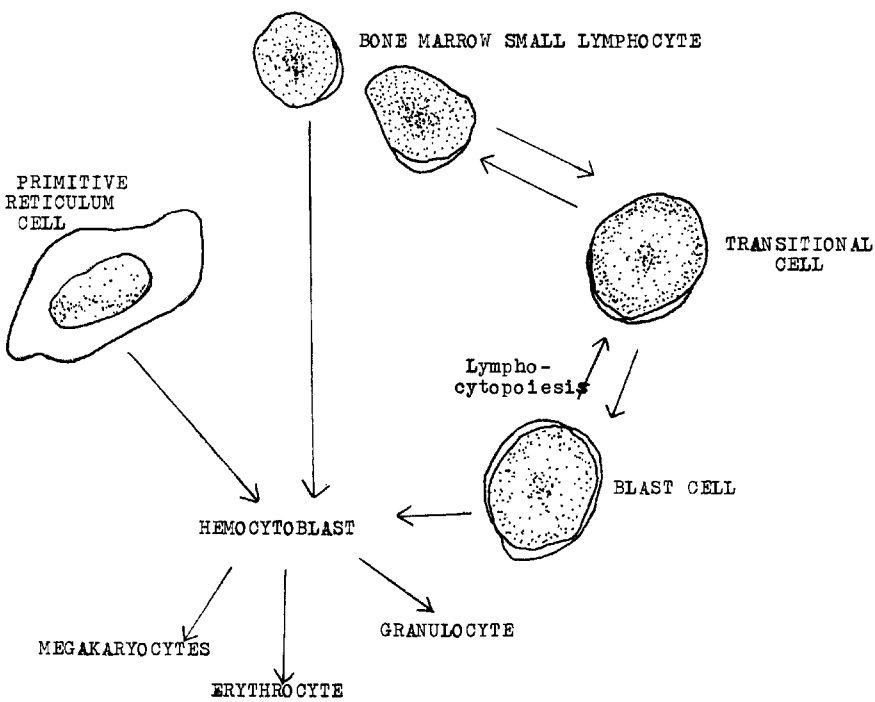

FIGURE 2. Modified monophyletic therory of blood-cell development.

cells (Maximow, 1924; Jordon, 1935; Bloom, 1940). The common parent cell was usually referred to as a pleuripotential stem cell, because it could differentiate into several kinds of mature forms. If these were the "black" and the "white" among theories describing hemopoiesis, there were many more which were shades of gray.

More recently, a unified concept has emerged, with a single alternate version. Elegant experiments designed to study the repopulation of bone marrow in irradiated animals have shown that implanted hematopoietic tissue will proliferate and form macroscopic colonies in the spleen of the irradiated recipient (Till and McCulloch, 1961). These colonies were derived from a single cell and contained myeloid, erythroid, and lymphoid elements (Becker, McCulloch, and Till, 1963). Tests of steroid hormone (testosterone) and stress effects on blood-cell production have provided strong evidence that erythroid elements and granulocytic leukocytes share a common origin. For example, testosterone treatment stimulates erythrocyte production in experimental animals, and at the same time, a decrease in granulopoiesis is evident. Erythropoietin, the hormone regulating red-cell production, exhibits a similar inhibition of granulocyte development. The relationship between these cells and the lymphoid series is less clear, but evidence points to transformation of lymphoid-like cells residing in the bone marrow into the myeloid lineage (Rosse and Yoffey, 1967; Yoffey, 1960).

The prevalent view is that erythrocytes and granulocytes come from a common stem cell, but follow different pathways toward differentiation. Lymphocytes may come from a different stem cell, but can transform into the granulocytic maturation sequence under certain conditions. The study of the kinetics of granulocyte formation would suggest that transformation is not the major source of granulocytic precursors and functions only when the demand for granulocytes is high. Post-natal lymphopoiesis is felt to be a result of the migration of some ancestor in the developing fetal bone-marrow to the thymus and other lymphoid structures (Wintrobe, 1969, p. 16). This would imply a common ancestor for all the blood cells and illustrates the still-existing confusion. This monophyletic concept does not appreciably alter our understanding of the process of proliferation and differentiation of granulocytes from some stem cell, regardless of its ultimate origin.

THE MYELOPOIETIC STEM CELL

The search for an identifiable stem cell has been one of the most fruitless but yet most important subjects in the study of hemopoiesis. As indicated above, the conclusive demonstration of the complete developmental lineage of a granulocyte without knowing the starting point is virtually impossible.

Several approaches to the problem have been attempted (fig. 3). Till and McCulloch's (1961) previously described studies of hemopoietic colony-formation in the spleens of irradiated rats have indicated the existence of a pleuripotential stem cell capable of giving rise to erythroid, myeloid, and lymphoid colonies. These colonies have been shown to arise from a single cell (Becker, *et al.*, 1963).

When mast cells or normal bone-marrow cells are cultured in tissue-culture medium partially solidified with agar, certain of the cells have the ability to form discrete colonies on the agar surface by repeated cell division (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). The colonies produced in this manner are primarily granulocytic, and in early work it was suggested that the colony-forming units (CFU) were analogous to the stem cells forming colonies in the spleen (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966).

The suggestion that the *in vitro* colony-forming cells are more differentiated than the CFU's identified in the spleen-colony assay has been made by McCulloch and Till (1970). The cells giving rise to *in vitro* colonies are primitive members of the granulocytic series, not the transplantable CFU, but more likely a committed

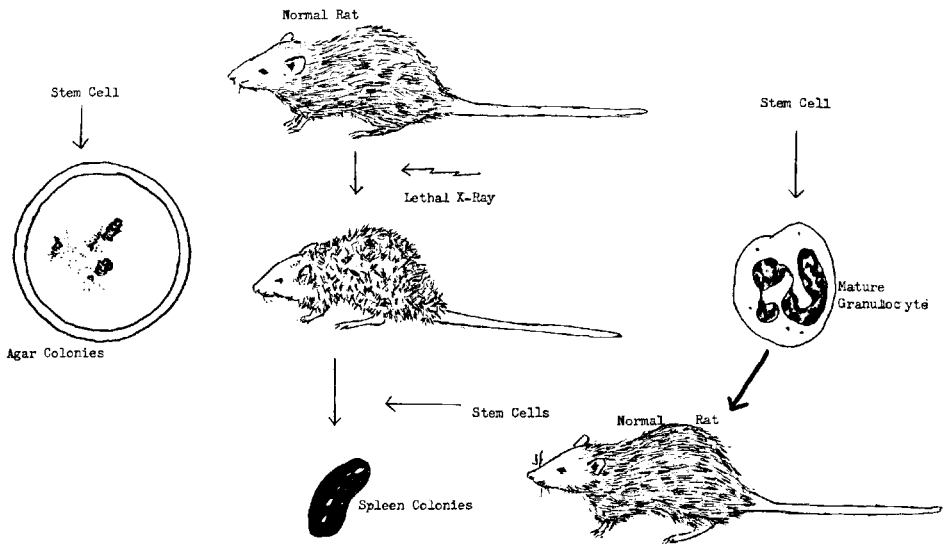

FIGURE 3. Various approaches to identification of the pleuripotential stem cell.

myeloid stem cell which has entered the differentiation cycle (Richard, *et al.*, 1970).

The question of whether either of these cells is the true primary stem cell in intact animals still exists. It may be that those cells which are able to form colonies in artificial essay systems are partially differentiated descendents of the *true* pleuripotential stem cell that begins the hemopoietic pathway in intact animals.

MYELOPOIESIS AS A SERIES OF COMPARTMENTS

The sequence of identifiable cell types in the maturation of granulocytes begins with a myeloblst form which possesses few of the morphologic characteristics of mature segmented granulocytes. The sequence terminating in a mature neutrophilic polymorphonuclear (PMN) leukocyte is illustrated in Figure 4.

Study of this sequence is facilitated if the process is divided into compartments with finite limits which can be easily measured. Boggs (1965) set forth such a compartmentalized scheme using the following divisions.

Stem Cell—primitive, highly undifferentiated (and unrecognizable) cells, which give rise to mature granulocytes. These might further be subdivided into committed and non-committed groups.

Mitotic—composed of cells which divide mitotically, corresponding to classical myeloblasts, promyelocytes, and myelocytes.

Maturation—those cells consisting of cells corresponding to metamyelocytes and ring, or stab, forms which have lost their capacity for mitotic division but which continue differentiation into mature granulocytes.

Storage—the bone-marrow reserve of segmented mature granulocytes (fig. 4).

KINETICS AND CONTROL

From a description of the pathways of granulocytic differentiation, it is useful to turn to the kinetics of the process of granulopoiesis. The process of differentiation from the stem-cell compartment is accompanied by proliferation through the myelocyte stage, but the maturation process progresses alone past this point (King-Smith and Morley, 1970). The transit time from stem cell to metamyelocyte

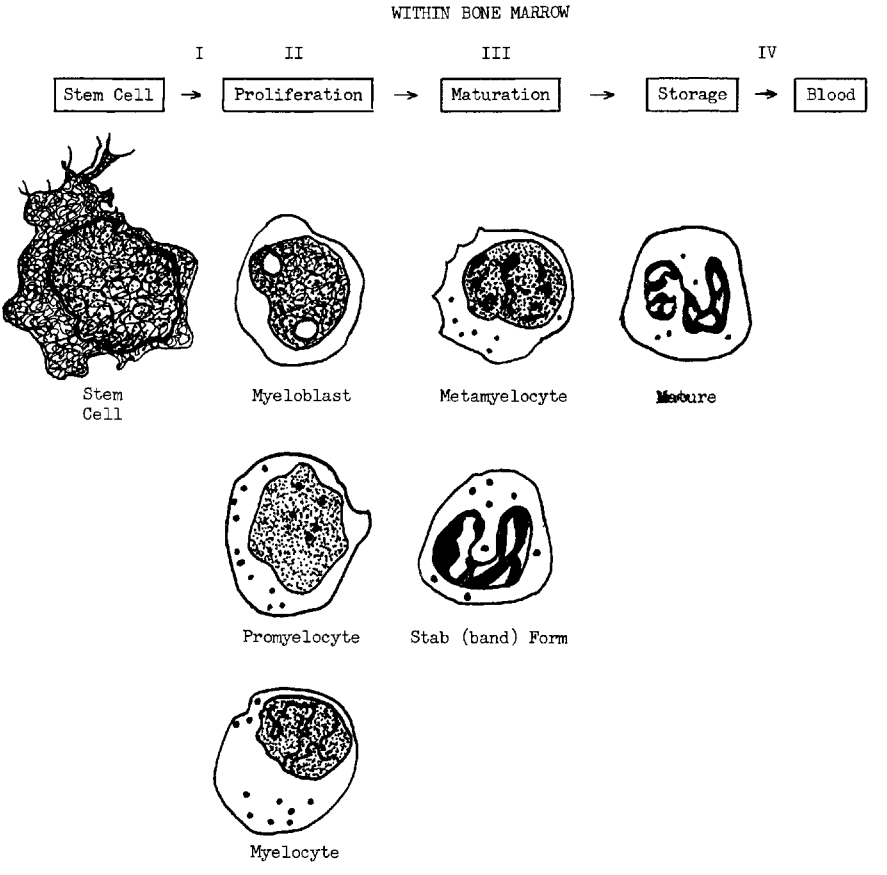

FIGURE 4. The compartmental scheme of granulopoiesis showing the cell types in each compartment and four levels where control factors might act.

(the proliferation compartment) is in the vicinity of 140 hours under normal conditions (Cronkite and Vincent, 1970). The minimum time needed to traverse the maturation compartment and the bone-marrow granulocyte reserve is 96 hours, with a maximum of 144 hours (Cronkite and Vincent, 1970). This establishes an obligatory bone-marrow transit-time on the order of 10 days, with an average of two and a half additional days spent in the marrow-reserve compartment (King-Smith and Morley, 1970). Movement of granulocytes through the maturation compartment is believed to be a "first-in, first-out" process (Maloney and Patt, 1968). Additionally, King-Smith and Morley (1970) estimate the marrow reserve as six times the total blood granulocyte pool, if it contains all of the mature granulcytes and the most nearly mature band forms.

The above information is derived from experimental measurements and from computer simulation of the process of granulopoiesis. This knowledge of cell kinetics has proved to be the key to predicting and to proving experimentally the existence of feedback-type control-mechanisms. Computer simulation of granulopoiesis has shown the probability that three feedback loops exist, each of which would regulate a particular phase of the entire process (King-Smith and Morley, 1970). These feedback loops may come directly from a sensor for the

number of a particular kind of granulocyte in the peripheral blood, or from the destruction of phagocytic cells in extra-vascular tissue. A more sophisticated feedback system might be postulated that would involve the neuro-endocrine axis with production of a specific hormone perhaps similar to erythropoietin (fig. 5). Little evidence exists for acceptance or rejection of any of these hypotheses.

It has also been possible to demonstrate three categories of stimulating factor acting on granulopoiesis. First, the leukocytosis-inducing factor (LIF) has been shown to promote release of cells from the marrow storage pool (Dornfest, *et al.*, 1962; Boggs, *et al.*, 1966; Katz, *et al.*, 1966). Secondly, a serum-protein factor stimulating proliferation of granulocytic precursors (Colony-Stimulating Factor, CSF) was demonstrated *in vitro* by Metcalf's group (Bradley and Metcalf, 1966; Stanley and Metcalf, 1969) and *in vivo* by our laboratory (Graham, Morrison and Toepfer, 1968; Graham and Morrison, 1970; Graham and McMahon, 1971).

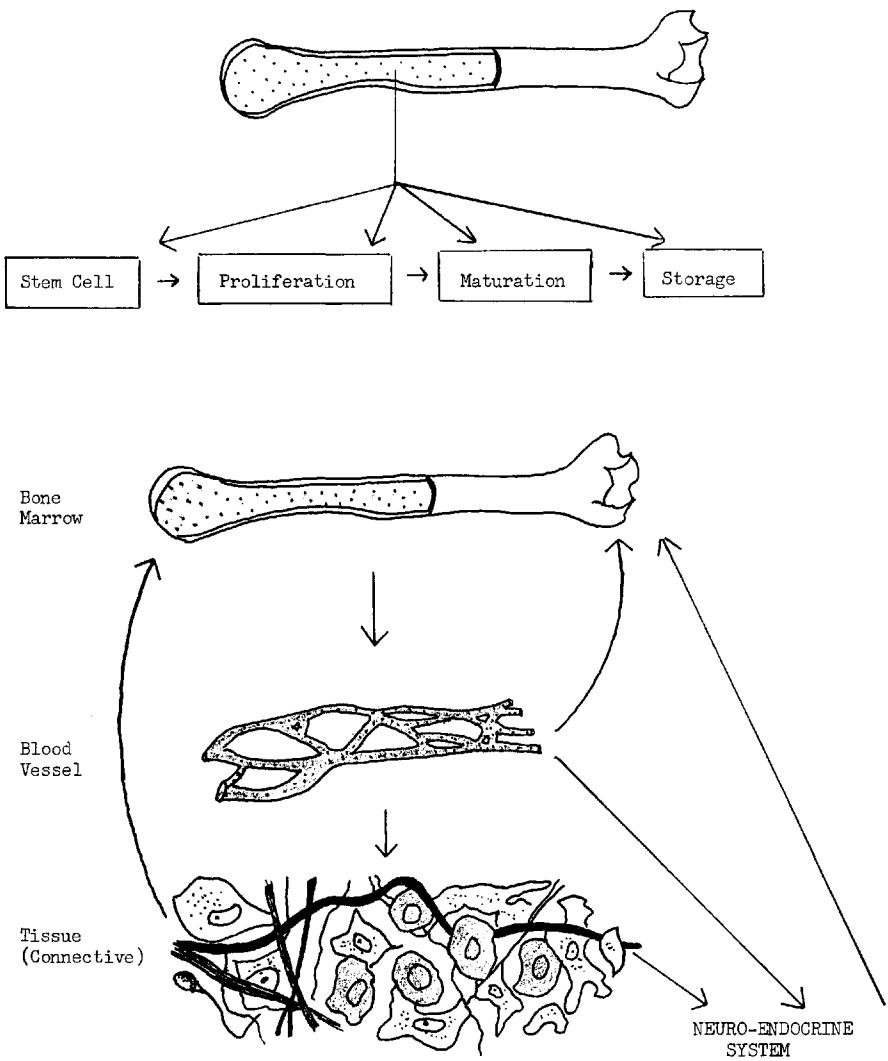

FIGURE 5. Possible feedback loops controlling stages in the development of granulocytes.

Finally, our laboratory has demonstrated the existence of a rat-serum macroglobulin which stimulates differentiation without significantly affecting the rate of proliferation (Graham, *et al.*, In prep.).

It should also be mentioned that a lesser known, but potentially more exciting system of inhibitory factors exists. The granulocytic chalone was discovered by Rhyomaa and his coworkers several years ago (Rytomaa and Kivinieme, 1968b). Metcalf has reported inhibition of colony-stimulating factor by a lipoprotein in the macroglobulin fraction of serum (Donald Metcalf, personal communication, 1971). Our laboratory has been working with a protein in the 120,000-800,000-dalton range which inhibits granulocytic development *in vivo* and repopulation of myelo-depleted bone marrow. The known properties of these factors are summarized in Table 1.

Thus, at the present time we have considerable knowledge of the granulopoietic pathway, its timing, and the control factors which regulate it. With this information it is possible to begin the study of abnormal granulopoeisis, such as myelogenous leukemia.

TABLE 1

Substances implicated in the control of granulopoiesis as of July 1972 and their known characteristics

I.	*Colony Stimulating Factor (Myelopoietic Factor)*—60,000 daltons, alpha$_1$ globulin, glycoprotein, stimulates proliferator.
II.	*Macroglobulin*—"alpha" macroglobulin; 800,000–1,200,000 daltons, glycoprotein, little effect on division *in vivo;* stimulates differentiation.
III.	*Release Factors*—reports of chemical nature varied, causes release of mature cells from marrow in response to stress.
IV.	*Chalone*—small non-protein; 4,000–12,000 daltons, inhibits mitosis.
V.	*Antichalone*—protein; 30,000–60,000 daltons; inhibits chalone effect.
VI.	*CSF Inhibitor*—lipoprotein in macroglobulin fraction, inhibits colony stimulating factor.
VII.	*Inhibitor "X"*—intermediate-size protein; 120,000–800,000 daltons, inhibits granulocytic development *in vivo;* and repopulation of marrow in myelo-depleted rats.

ALTERED CONTROL MECHANISMS IN MYELOGENOUS LEUKEMIA

In the introduction to this paper, the reasons for stressing the investigation of the mechanism controlling the development of granulocytes and its role in the production of the leukemic state were discussed. The transmission of most leukemia viruses from parent to offspring in a "vertical" type of infection, and the probability of resulting development of specific immunologic tolerance of the foreign viral antigens, mean that conventional vaccination is unlikely to be effective. Also, Metcalf (1971) has succinctly pointed out that leukemic cells may not be autonomous cancer cells and that the disease process in some types of leukemia may be reversible (fig. 1). He suggests that the virus may affect production of control factors rather than permanently alter the cells.

An attempt has been made in our laboratory to analyze this hypothesis, using murine myelogenous leukemia as a model system. The C57B1/6J inbred mouse strain is susceptible to the C1498 transplantable myelogenous leukemia, although it has a very low incidence of spontaneous leukemia. The C1498 tumor may be maintained in the C57b1/6J mouse strain by implantation of a suspension of the tumor cells subcutaneously into the axillary region of the recipient mouse. Following a latent period of three to six days, a tumor develops at the site of implantation, along with a frank leukemia in the blood and blood-forming (bone marrow, spleen, etc.) organs of the mouse. The tumor is transplantable with 100 percent success and results in death of the recipient in an average 13.9 days established in over 200 trials in our laboratory. It should be noted that murine myelogenous leukemis is an acute form of the disease in which the first hematologic symptoms

appear on the fifth day post-implantation and few, if any, remissions occur before death follows, during the 10th through 16 days. A detailed study of this leukemia has been completed and is in the process of publication (McMahon and Graham, In prep.).

A test group of 20 mice was used in each experiment, along with suitable control groups sham injected with saline or with bovine albumin. The control substances and antisera evaluated were given in a quantity previously demonstrated to produce statistically significant changes in the bone-marrow cytology of normal albino rats. (Graham, Earney, McMahon, and Tjan, 1972; Graham, McMahon, Earney, and Tjan, In press). For the factors, this was 40 mg per kg body weight.

Two basic approaches have been attempted to learn whether the production of granulopoietic control factors is defective in murine myelogenous leukemia or whether the leukemic cell is insensitive to the normal control substances. First,

TABLE 2

Summary of data from preliminary experiments testing effects of granulopoietic-control substances on murine myelogenous leukemia

TWELVE DAYS

Post-Tumor Implantation

Survival (days)		Leukocyte ($X10^3$)		Hematocrit (%)	
Leukemia	13.9	Leukemia	7.27	Leukemia	33.9
Leukemia+BSA	13.4	Leukemia+AMF(A)	8.62	Leukemia+MF	37.3
Leukemia+MF	14.3	Leukemia+"X"	6.37	Leukemia+AMF(A)	38.3
Leukemia+AMF(A)	14.6	Leukemia+SHAM	5.42	Leukemia+SHAM	41.9
Leukemia+SHAM	15.5	Leukemia+MF	4.89	Leukemia+BSA	42.8
Leukemia+"X"	16.3	Leukemia+AMG	4.63	Leukemia+MG	43.6
Leukemia+MG	16.3	Leukemia+MG	3.82	Leukemia+AMF(B)	44.3
Leukemia+AMF(B)	18.7	Leukemia+AMF(B)	3.73	Leukemia+"X"	45.8
Leukemia+AMG	18.8	Leukemia+BSA	3.36	Leukemia+AMG	50.0

EIGHTEEN DAYS

Post-Tumor Implantation

Red Blood Cell ($X10^6$)		Leukocyte ($X10^3$)		Hematocrit (%)	
Leukemia+AMF(B)	3.12	Leukemia+AMF(B)	3.80	Leukemia+AMG	39.7
Leukemia+BSA	3.45	Leukemia+AMG	5.17	Leukemia+"X"	38.3
Leukemia+MG	4.08	Leukemia	5.18	Leukemia+MF	34.5
Leukemia+AMF(A)	4.08	Leukemia+MG	5.18	Leukemia+SHAM	33.8
Leukemia+SHAM	4.23	Leukemia+"X"	6.63	Leukemia	32.1
Leukemia+AMG	4.37	Leukemia+AMF(A)	6.25	Leukemia+AMF(A)	30.0
Leukemia+MF	4.56	Leukemia+BSA	7.45	Leukemia+BSA	29.8
Leukemia	4.87	Leukemia+SHAM	7.72	Leukemia+MG	29.0
Leukemia+"X"	5.72	Leukemia+MF	8.85	Leukemia+AMF(B)	26.0

a treatment regimen was established, beginning with the first appearance of leukemic symptoms. The different control factors were injected every other day. Later, injections were increased to a daily schedule, with double the dosage of factor.

Secondly, an effort was made to protect mice against the leukemia by administering the control factors before injection of tumor cells. Preliminary experiments revealed that prophylactic administration of the control substances produced no change in the disease course, except for a prolonged survival of mice injected with the low-molecular-weight myelopoietic-factor (CSF).

In assaying the effects of injections of stimulatory or inhibitory factors, survival time and peripheral leukocyte, erythrocye, and hematocrit values were initially followed. We have since added the parameters of organ and tumor pathology and of bone-marrow cytology in order to have the most sensitive measure of the effect of a control-factor injection on the disease course.

Early studies (Table 2) have demonstrated that antiserum to the low-molecular-weight myelopoietic-factor is effective in extending the survival of mice implanted with the C1498 tumor if administered prior to tumor implantation. It has little or no effect on the disease course if given after tumor injection. The antiserum has little effect on hematologic values.

Alternate-day therapy with the macroglobulin factor (Table 2) effectively extends survival time and maintains the normal erythroid-leukocyte ratio in the peripheral blood over the first 12 days post-tumor implantation. Anemia is prevented during the same period and the animals remain "healthy" in appearance and behavior.

Antiserum to the macroglobulin fraction injected on alternate days produced similar results, with increased survival and delayed development of anemia and elevated leukocyte counts (Table 2). The percentage of segmented granulocytes in the peripheral blood was markedly lower in anti-macroglobulin-treated rats than in untreated leukemia animals.

Similar studies of the effects of the inhibiting factor "X", being carried out at the present time, suggest similar maintenance of normal hematologic values without prolonged survival, but are incomplete. The pathologic and bone-marrow studies are also in progress.

Anti-myelopoietic factor, given prophylactically, works in inhibiting the normal myelopoietic factor and maintaining erythroid-myeloid balance, whereas anti-myelopoietic factor therapy does not. This supports the hypothesis either that leukemic animals no longer have a functional myelopoietic factor or that the factor is altered in such a way as to be immunologically unrecognizable. Both the macroglobulin factor and the antiserum to it affect the disease course, indicating that this particular control factor is not substantially altered by the viral genome inducing the disease process.

These results suggest that the leukemic state is due to an alteration in the normal control mechanism and not to a permanent alteration of the cells. If these findings are born out in further studies, myelogenous leukemia stands in direct contrast to most forms of neoplasia, where cells are permanently transformed. Other laboratories have already suggested that this is the case for human myelogenous leukemia. These preliminary results serve not only to suggest a new direction for cancer research, but also to illustrate the need for understanding development processes. Further studies will indicate the significance of control-factor alterations in the mechanism of myelogenous leukemia.

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