Productivity in Normal and Leukemic Granulocytopoiesis*

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1. Introduction

At the request of the organizers of this workshop on "Modern Trends in Human Leukemia II" we have been asked to review briefly some aspects of the physiology and pathophysiology of myelopoiesis, focusing mainly on the problems of the obvious deficiency of this system in case of acute myelocytic leukemia to provide an adequate number of granulocytes. A vast amount of information has been collected during the last 1 or 2 decades on the possibilities and limitations of cell production and differentiation in normal and leukemic myelopoieses. In spite of this, we have to confess today that there are many more open questions than solved problems. It probably is correct to state that "we are still quite ignorant about normal and leukemic cell production and differentiation but at a higher level" than 17 years ago, when the first cell kinetic study utilizing tritiated thymidine as a specific DNA label was performed in Dr. Cronkite's laboratory (1, 2, 3).

It is therefore the purpose of this presentation to outline the present concept of normal and leukemic cell proliferation and differentiation using granulocyte kinetics as a model. This will lead to the conclusion that the obvious deficiency of granulocyte production in acute leukemia is a consequence of a highly ineffective cell proliferation and differentiation in the appropriate precursor compartments and points to the stem-cell pool as the major site of leukemic cell transformation.

2. Efficient granulocyte production: a property of the normal granulocytic cell renewal system

The normal granulocytic cell renewal system (Fig. 1) maintains in the peripheral blood of man a granulocyte concentration that appears to be constant from day to day, although detailed studies may indicate a cyclic pattern with a phase length of some twenty days, the amplitude of which may increase in certain diseases such as cyclic neutropenia (4, 5). The extravascular portion, the function of which guarantees a sufficient blood granulocyte concentration, is normally located ecxlusively in the bone marrow, distributed in many bones throughout the body, but nevertheless acting as one organ. The regulatory mechanisms responsible for this unity
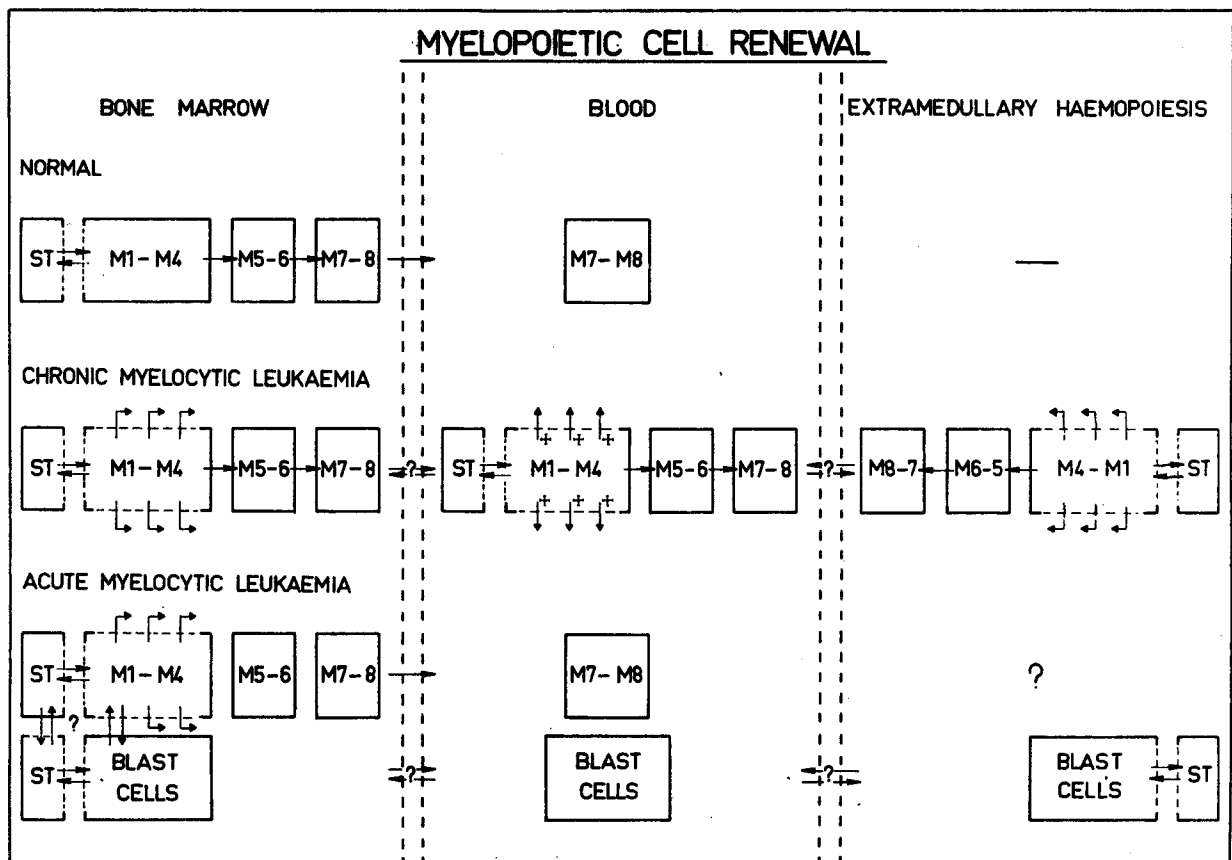
Fig. 1: Schematic representation of the functional structure of the myelopoietic cell renewal system in normal conditions, in chronic myelocytic leukemia and in acute myelocytic leukemia.

of function, in spite of topographic diversity, are far from being understood in detail but should include humoral as well as neural factors and may well be associated with stem-cell migration via the blood stream. The functional structure of the granulocytic cell renewal system can be described as a number of catenated cell compartments. The granulocytes of the blood – segmented forms and band forms, M 8 and M 7 respectively, – represent the *functional pool* of cells. It is known since the work of the Salt Lake City Group (6) that the half-life of the mature cells is in the order of 7 hours. The mature granulocytes leave the blood either by emigration or after death due to senescence (7, 8). This pool is fed continuously by the M 7/M 8 *storage pool* in the marrow. This is considered to be a part of the *maturation-only pool* in the marrow, but with a variable "time delay": it is known through the work of the Brookhaven Group that the time of granulocytes between the last division of myelocytes and their release into the blood as mature granulocytes may vary and can be as short as 2 days (instead of 4 days) in cases of infection (7, 8). It is this pool that contains a large amount of reserve cells that can be released into the blood after appropriate stimulation and may increase the blood granulocyte concentration several times within a few hours (9). This pool in turn is fed by the *maturing-only pool* of metamyelocytes and juvenile cells – M 5 and M 6. Furthermore, there is a *dividing-maturing pool* of granulocyte precursors, the myeloblasts, promyelocytes and myelocytes (M 1 – M 4). These cells are considered

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to be capable of division but not of self-replication. On the basis of morphological, cell-kinetic and cinematographic studies, the number of cell divisions in this pool has been estimated to be 2–4 in normal marrow (10–13). The maintenance of a homeostatic equilibrium between production and utilization in this system is maintained by a *stem-cell pool* which has the dual function of maintaining its own size and at the same time responding to specific stimuli, such as erythropoetin or a still hypothetical granulopoietin (14) (which may or may not be identical to CSF (15), with differentiation into the well-known hematopoietic cell lineages. Dr. Cronkite, in this conference, has indicated the present state of knowledge about this cell pool and its conceptual difficulties (16). Nevertheless, it appears justified to assume that this pool contains at least 2 sub-populations of cells which may be denoted as "committed and uncommitted", or "determined and undetermined" (14, 17, 18), depending on the author. These expressions are meant to indicate that this pool contains cells that have to undergo a certain process of physiological "development" or "maturation" in order to proceed from a pluripotent stage – in which most cells appear to be in a cytokinetically resting phase called G_0 (19) – to a stage of being "committed" to respond to specific stimuli with irreversible differentiation, resulting in a catenated process of cell multiplication and/or maturation. In the human, various methods have been developed in recent years to elucidate one or the other aspect of this pool. In diffusion chambers implanted into irradiated recipients (goats (20), rats and mice (21, 22)), human bone marrow cells and blood mononuclear cells have been shown to be able to form granulocytes, erythroblasts and megakaryocytes and, hence, to be indicative of the presence of a population of pluripotent cells. In cell cultures with appropriate media and stimulation factors, one has been able to trigger stem-cells into granulocytic, erythropoietic and megakaryocytic differentiation, thus looking – presumably – at the "committed" cell population of the stem-cell pool (23, 24). In spite of such efforts, it appears that it is not yet possible to characterize the stem-cell pool completely in terms of quantitative and qualitative properties, but one is "recognizing" only certain aspects and is limited by the inherent constraints of the methods used to approach the problem. It may be of interest at this point to say a few words about the regulatory mechanisms of the granulocytic cell-renewal system. Its particular structure led several investigators to the hypothesis that it can be considered as a cell system, regulated by a negative feed-back mechanism and that it must have oscillatory properties (25, 26). It is assumed that there are factors that are capable of inducing a release of granulocytes into the blood stream in case of need and that there are other factors, both inhibiting as well as stimulating factors, that trigger cells into differentiation at the stem-cell level or prevent them from differentiating (27, 28, 29).

In *conclusion*, the characteristic blood granulocyte concentration appears to be the result of a feed-back controlled cell-renewal system. This is capable of life-long granulocyte production without exhaustion and can adapt itself to increased demands by an appropriate increase in production. Further research has to explore the degree of efficiency under the conditions of the normal steady state. A maximum degree of efficiency would be reached when all cells triggered into the granulopoietic pathway undergo an equal number of cell divisions and all reach the blood as mature cells without cell death along the dividing-maturing pathway by intrinsic deficiencies. It may well be, however, that there is normally a "death func-

tion" at all levels of granulopoietic proliferation and maturation, the extent of which would be highest in the functional cell pool (30).

3. Inefficient granulocyte production: a consequence of leukemic transformation of the granulopoietic cell system

In leukemia, both in the chronic myelocytic and in the acute myelocytic forms, the granulocyte production is drastically altered (Fig. 1). In *chronic myelocytic leukemia* (CML), one observes in the blood and in extramedullary sites the presence of granulocyte precursors. In a recent article, Drs. Vincent and Cronkite and associates presented a wealth of information on the cell kinetics of this disease and came to the conclusion "that increased myelocyte proliferation as well as an increased stem-cell input must contribute to the expansion in granulocytopoiesis seen in chronic myelocytic leukemia. Myelocytes in CML divide 3–4 times, compared with twice in normal marrow, thus increasing the amplification of the stem-cell input. The size of the total myelocyte mass in the patients studied was estimated to be 3 and 25 times normal (12)". Further studies have to explore the extent of cell death of the myelocytes so produced and hence the degree of efficiency or inefficiency of cell production in the system.

In *acute myelocytic leukemia*, the blood picture is characterized by the presence of some mature granulocytes and of "blast-cells" (Fig. 1). There is evidence in the bone marrow of some proliferation and maturation of myelocytes and promyelocytes, resulting in the appearance of some normal-looking and functioning granulocytes in the blood. However, the bulk of cells usually is comprised of "blast-cells" showing a spectrum of morphological appearance. There is also today a fair amount of information on the kinetics of such blast-cells in bone marrow and blood and, more recently, some evidence about the developmental potential of the leukemic blast-cells. Usually, the blast-cells in the bone marrow show a low tritiated thymidine (^{3}H-TdR) labeling index as compared to normal myeloblasts or promyelocytes, when exposed to ^{3}H-TdR in vitro or in vivo. The labeling index of blood blast-cells is still lower (31). If leukemic blast-cells are labeled in vitro with tritiated cytidine (^{3}H-Cyt) and autotransfused, the calculated blood transit times are between *3.7 and 8.5 days*, much longer than those of granulocytes (32). Hoelzer and Kurrle in our group (33) have studied the fate of leukemic blast-cells in diffusion chambers implanted intraperitoneally into irradiated mice. They came to the conclucion that some leukemic blast-cells appear to have the potential to differentiate into granulocytic precursors and to mature into granulocytes. Thus, it may well be that the accumulation of blast-cells in human acute myelocytic leukemia can be taken to indicate the extreme of inefficiency: the bulk of cells accumulates in the form of "blast-cells" that, in principle, may have the potential for differentiation and production of granulocytes, but rarely do so in the phase of full-blown acute leukemia. It is, therefore, of interest to ask whether there is any normal rest function of granulocytopoiesis and, if so, with what characteristics, or whether granulocyte production arises from leukemic precursor cells, some of which exercise their potential to differentiate, proliferate and mature.

In many patients, both in Brookhaven and in Ulm, the kinetics of granulocyte production was studied by means of tritiated thymidine labeling (7, 8). The typical,
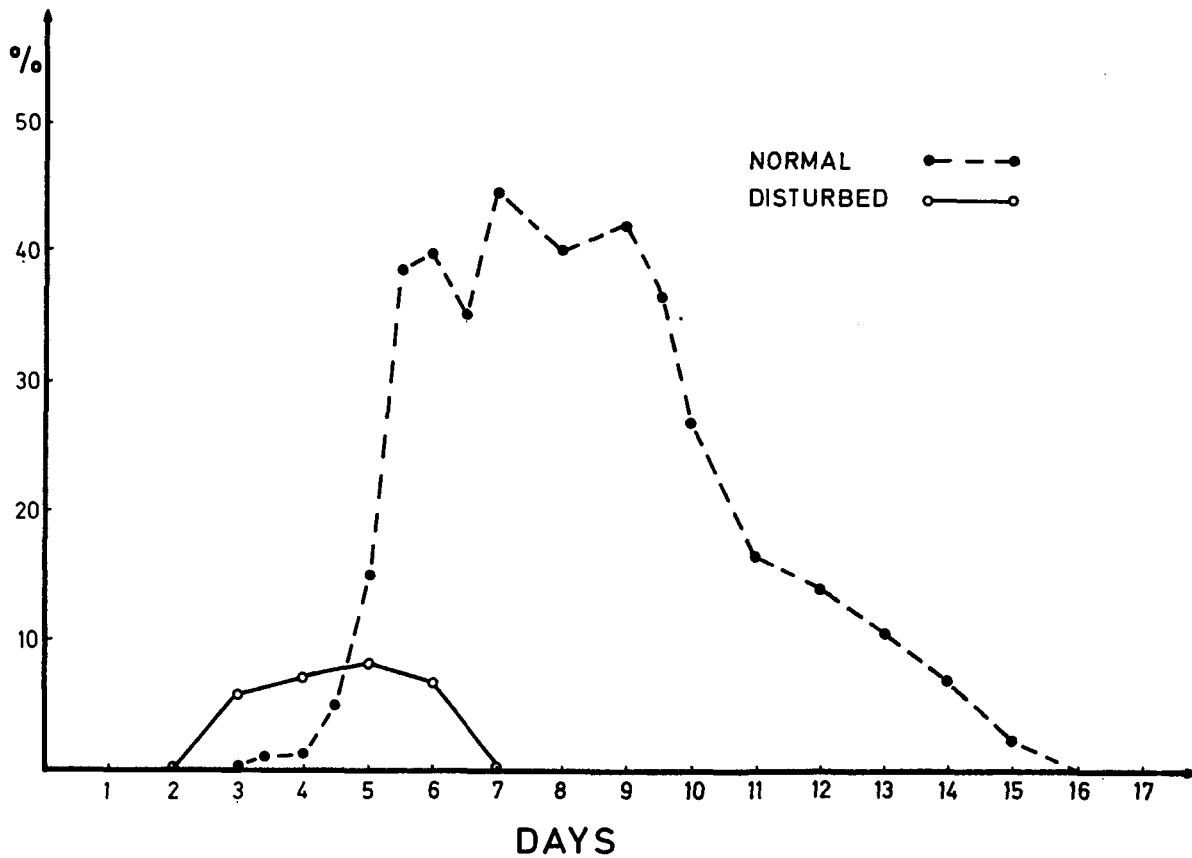
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LABELING INDEX OF GRANULOCYTES

Fig. 2: Labeling index of blood granulocytes as a function of time after administration of 3H-TdR.
○ person with undisturbed hemopoiesis.
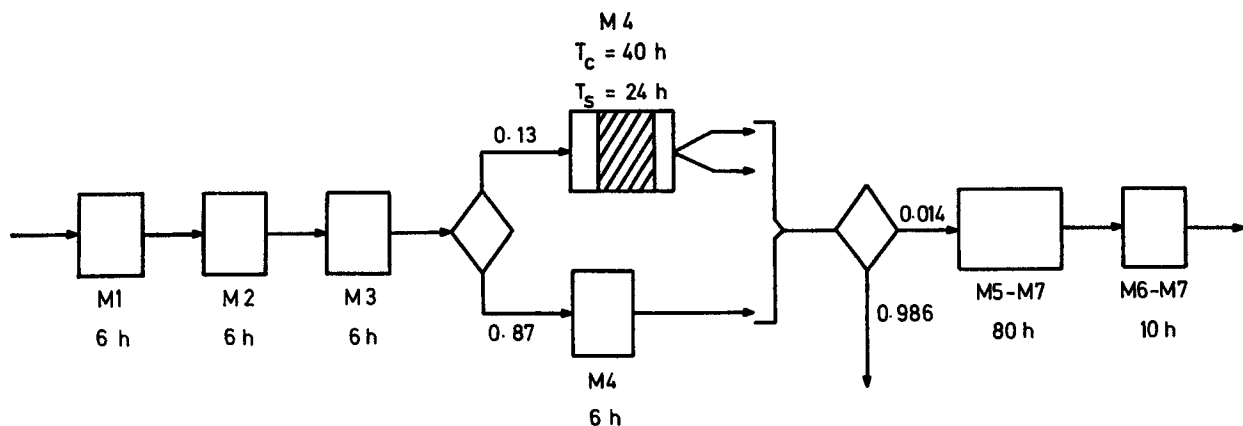● patient with acute myelocytic leukemia.

normal labeling pattern of blood granulocytes after thymidine labeling shows the appearance of the first labeled, segmented forms in the blood after a lag-phase of about 4 days (maturation time from the last division to release) followed by a rise of the labeling index to about 60 % and a subsequent decline (Fig. 2). The same labeling pattern is seen for granulocytes removed from the mucous membranes, indicating that they had migrated onto these surfaces, and for the pycnotic granulocytes, the latter delayed, however, by some 24–30 hours, indicating the upper limit of life in the blood stream. In various forms of acute leukemia, the labeling pattern of blood granulocytes in acute leukemia is markedly different. Although the first appearance of labeled granulocytes may be normal or somewhat shortened – as seen also in cases of infection – there is a labeling pattern with several abnormal features. The labeling indices never reach the normal values but are below 30 %, in many cases not exceeding 10–20 percent. The labeled cells disappear again quite rapidly, so that, after 6 days, many or all may have disappeared. In other cases, a few may be seen until 12 days after ³H-TdR injection (7, 8).

The attempt to answer the question as to the reasons for the low labeling indices combined, usually, with a granulocytopenia in spite of a normal blood emergence time for labeled granulocytes, leads one to the problem of the efficiency or

inefficiency of cell production and/or maturation in the various precursor pools of blood granulocytes.

In Fig. 2, the labeling pattern of blood granulocytes is shown for a patient with undisturbed hemopoiesis (34) and for a patient with acute myelocytic leukemia. It is clear that the labeling pattern of the leukemic patient is markedly different with respect to the maximum labeling index achieved and to the duration of the presence of labeled cells. In order to try to formulate questions for further studies on the efficiency of cell production in leukemic patients, we approached the problem by trying to simulate the labeling curve on a computer using the GPSS (general purpose simulation system) language (35). In order to simulate the labeling curve, the following experimental data had to be recognized (one patient):

1. The labeling index of the bone marrow cells (M 2–M 4) 1 hour after ³H-Tdr was found to be 14 %.
2. The relationship of the relative proportion of the dividing-maturing (M 2–M 4) cells to the maturing-only cells (M 5–M 7) and hence, the absolute numbers of these cells, was 18:1.
3. The DNA-synthesis time of the dividing cells was estimated to be 24 hours.
4. In the circulating pool of granulocytes, there were 123 cells per µl.

DISTURBED GRANULOPOIESIS

Fig. 3: The model of the granulocytic cell renewal system in a patient with AML.

A model that allows the approximate reconstruction of the labeling pattern of blood granulocytes in this patient with acute leukemia and still recognizes all the conditions specified in points 1–4 is given in Fig 3. In this model, one has to assume that cells entering the M 1, M 2 and M 3 compartments do so without synthesizing DNA and dividing. The compartment transit time must be taken to be 6 hours for each. After leaving the M 3 compartment, a divergence of the cell "stream" occurs. About 87 % of the cells enter an "ineffective" M 4 compartment which they leave after 6 hours of further maturation. The remaining 13 % of cells coming from M 3 synthesize (in M 4) DNA and divide. The DNA synthesis time was determined to be 24 hours, the cell cycle time 40 hours. In such a system, a labeling

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index of 14 % is found. In order to find now a relationship of 18:1 between M 2–M 4 cells and M5–M 7 cells and in order to fill the circulating compartment with 123 granulocytes per µl, 98.6 % of the cells coming from the two M 4 compartments must leave the system (by cell death) and only 1.4 % of the cells enter the maturation-only compartment to stay in it for an average of 80 hours. The functional pool, with a transit time of 10 hours, and a marginal and circulating pool of equal size is the last compartment of such a simulated system. To maintain a concentration of 246 granulocytes per µl in the last compartment an efflux-rate of 1580 cells/h from the stem-cell pool must be provided.

As already stated, the computer language used was the GPSS. The particular advantage of this language is seen in the fact that the inevitable variabilities of experimental data can be fully recognized.
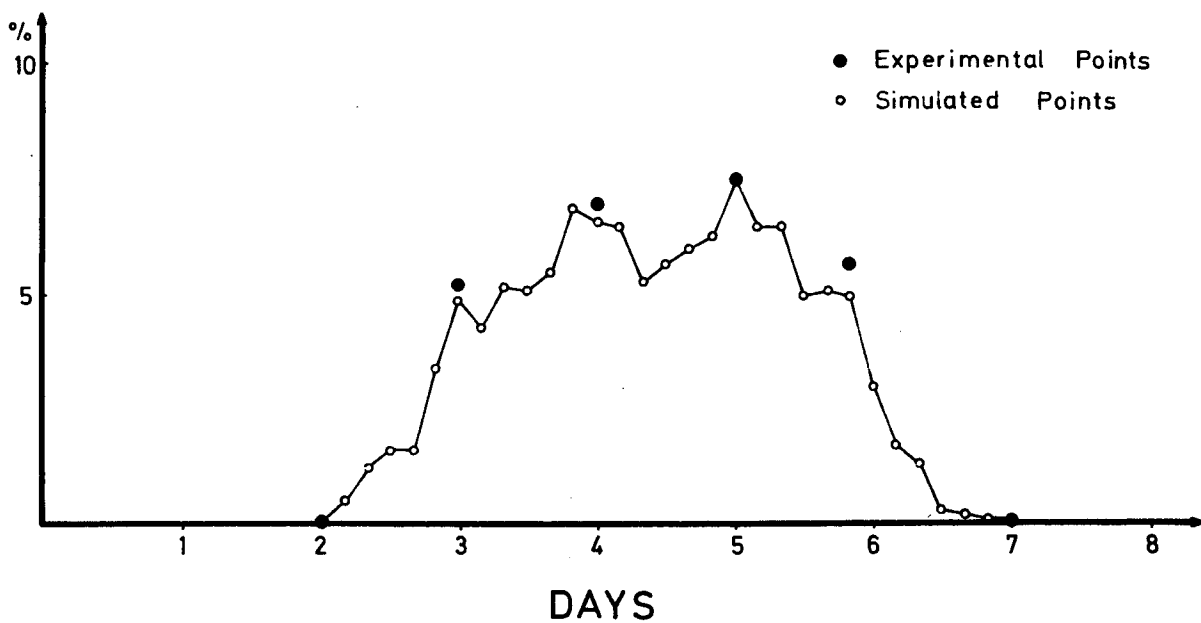
LABELING INDEX OF GRANULOCYTES

Fig. 4: The computer simulated labeling index in the functional pool in comparison to the experimental points.

Fig. 4 shows the labeling index pattern in the functional pool as it was simulated by the computer system in comparison to the experimental points. There is evidence that there is reasonable agreement between the experimental points and the simulated curve. In the simulation system, the time parameters of the system were assumed to follow a normal distribution with a standard error of 50 % of the mean. It was assumed that there is a steady state for the duration of the experimental study.

In order to obtain the labeling indices as shown in Figure 4 and with recognition of conditions 1–4 (see above), several mean values for the different system-parameters were obtained and are given in Table 1.

Of course, the information obtained from computer system simulation on one patient is far from being sufficient to draw general conclusions. Furthermore, results of system simulation models cannot be taken as evidence that they reflect

Table I: Computer derived cell kinetic parameters in the patient with acute myelocytic leukemia

Time of cell cycle	40 h
Compartment transit time (M 1 – M 4)	6 h
Fraction of maturing cells	1.4 %
Kinetic (Stem-Cell pool)	1580 h^{-1}
Multiplication factor M 1 – M 4	1.13

4. Leukemic granulocytopoiesis: a consequence of inefficient productivity

Of course, the information obtaine from computer system simulation on one patient is far from being sufficient to draw general conclusions. Furthermore, results of system simulation models cannot be taken as evidence that they reflect biological facts, processes or dynamic events. They can help, however, to pose questions and to focus on experimental approaches for obtaining information on the biological mechanisms underlying the observed sequence of events.

In the present system-simulation of one patient with acute leukemia, it appears of interest to note that the labeling pattern observed points to several possible deviations from normal in the dividing-maturing pool of granulocyte precursors. First of all, there appears to be decreased degree of proliferation; the cells, originating in an undefined stem-cell pool, cannot undergo the normal sequence of doubling divisions. The presence of leukemic blast-cells in marrow and blood may thus be regarded as an accumulation of cells that cannot make full use of their potential to divide and mature. The facts that some of them can be labeled by means of tritiated thymidine and that they show a turnover – when studied after retransfusion, labeled with ^{3}H-cytidine – are indications of some renewal which, however, remains totally inept with respect to granulocyte formation. The information obtained from leukemic patients with respect to granulocyte turnover, on the other hand, shows that some granulocyte formation is possible. It is to be asked whether this granulocyte formation is a reflection of a resting of normal hemopoiesis or whether it is the inefficient responce of a leukemic blast population for differentiation and maturation (as may be observable in diffusion chambers) or both. The results of the simulation model could be interpreted to mean an overwhelming disturbance of the normal process of proliferation and maturation and would be more compatible with the notion of a leukemic cell population with a resting or maturing-only capacity. In other leukemic patients, the granulocyte labeling pattern resembles more that of a normal hemopoiesis. Thus, it may well be that the clinical diversity of hematological findings is due to the wide spectrum of differentiation potentialities that may be presented in leukemia: at the one end of the spectrum may be seen a complete block of differentiation for granulocyte formation and an accumulation of "blast-cells" unresponsive to differentiation signals; at the other end (in remission) may be a "leukemic" population that is practically normal in its capability to differentiate, to proliferate and to mature. Between these two extremes may be all degrees of lack of differentiation, proliferation and maturation; certainly, in the overt cases of leukemia, there is a high degree of inefficiency of gran-

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ulocyte production and, at best, an abortive attempt to respond to the demands of the periphery in terms of increased granulocyte production. The data obtained from the granulocyte kinetic study of leukemic patients also indicate a lack of responsiveness for differentiation at the level of the stem-cell pool. Normally, granulocyte removal from the blood results in a feed-back mechanism resulting in an increase in cell production. In leukemia, the demand is clearly there, but the appropriate proliferative pool is not able to respond with normal production and maturation.

From all these considerations, it may be concluded that the basic defect in leukemia must be sought in the stem-cell pool. It is here that one must locate a defect in the normal response to differentiate into a granulocyte lineage with subsequent proliferation and maturation. One must ask whether there is a complete transformation of all normal cells into leukemic cells without a residual normal population (even though suppressed) or whether there is a normal population remaining in conjunction with the leukemic population with a certain "growth advantage" which is, however, insufficient to produce normal cells.

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