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# Early Events in the Suppression of Myeloid Leukemic Cells by Biological Regulators *

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Culture of mouse myeloid leukemic cells in the presence of a variety of chemical substances can induce the differentiation of some at least of these cells to maturing granulocytes and macrophages. Essentially similar events occur with the human myeloid leukemia line HL60 [1].

Some of these agents, e.g., RNA and DNA inhibitors, are already in clinical use in the treatment of myeloid leukemia, and it may be therefore that part of their action in inducing remissions is not simply by their cytotoxic effects as has been assumed. Karyotypic evidence strongly suggests that, in remission, the repopulating hemopoietic cells are derived from normal precursor cells. However, it is quite possible that during the complex process of remission *induction*, enforced differentiation may play an important role in suppressing the previously dominant leukemic population.

With this increasing awareness of the complexity of action of the process of remission induction, it may be that other chemical agents known to enforce differentiation, e.g., the retinoids, will be tested clinically for their effectiveness as adjuvants in the induction of remission.

## A. Normal Regulation of Leukemia Cell Differentiation

Parallel experiments have shown that materials of biological origin are also capable of inducing differentiation in myeloid leukemia cell lines. Without detracting from the potential value of extrinsic chemical substances in controlling leukemia populations, it seems of importance to understand the chemical nature and source of these naturally occurring differentiation-enforcing materials, their function in normal health, and the nature of their action on leukemic cells.

Analysis has shown that in the mouse, three families of specific macromolecules control the proliferation of normal granulocyte-macrophage precursor cells. These are (a) M-CSF of molecular weight 70,000 or 49,000, with a proliferative action essentially restricted to macrophage precursors, (b) GM-CSF of molecular weight 23,000 but of at least two antigenic subtypes with a proliferative action on both granulocytic and macrophage precursor cells, and (c) G-CSF of molecular weight 25,000 with an action primarily but not exclusively on granulocytic precursors.

From the evidence available at present, these same molecules are involved in all experiments that have demonstrated the induction of differentiation in mouse myeloid leukemia cell lines using biological materials. However, the three subtypes of GM-CSF differ sharply in their ability to induce differentiation even using a cell line such as WEHI-3B, which, being a myelomonocytic leukemia, is potentially able to differentiate into either the granulo-

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| Dilution of G-CSF | Mean number of normal marrow colonies | Percent differentiated WEHI-3B colonies |
|---|---|---|
| 1:1 | 27±1 | 100± 0 |
| 1:2 | 25±1 | 100± 0 |
| 1:4 | 26±1 | 99± 1 |
| 1:8 | 28±1 | 99± 1 |
| 1:16 | 30±1 | 98± 3 |
| 1:32 | 25±4 | 86± 0 |
| 1:64 | 14±2 | 64±18 |
| 1:256 | 7±1 | 18±16 |
| 1:512 | 4±1 | 19± 2 |
| Endotoxin serum 1:6 | 83±4 | 100± 0 |
| Saline | 0 | 8± 6 |

**Table 1.** Activity of purified G-CSF (DF) in stimulating colony formation by normal marrow cells and differentiation of WEHI-3B leukemic colonies

Assay cultures of 75,000 C57BL marrow cells or 300 WEHI-3B cells contained 0.1 ml of varying dilutions of G-CSF purified from mouse lung conditioned medium. Cultures scored after 7 days of incubation. Control positive cultures contained 0.1 ml of a 1:6 dilution of C57BL post-endotoxin serum

cytic or macrophage pathway. Thus M-CSF appears to be completely inactive in enforcing differentiation; purified GM-CSF does exhibit detectable differentiating activity, primarily into the macrophage pathway; and G-CSF is highly active, forcing cells to enter either the granulocytic or macrophage pathway depending on the experimental conditions used [11, 12]. The high activity of G-CSF in enforcing differentiation has led to the introduction of a second name for this molecule, differentiating factor (DF). It needs to be emphasized, however, that the molecule is a proliferative factor for normal granulocytic precursors and is therefore a G-CSF [15]. Reports [6] that this factor acts only as a differentiation factor and not as a proliferative stimulus are technically erroneous.

Titration of purified G-CSF has shown that the dose-response curves for stimulation of colony formation by normal mar-

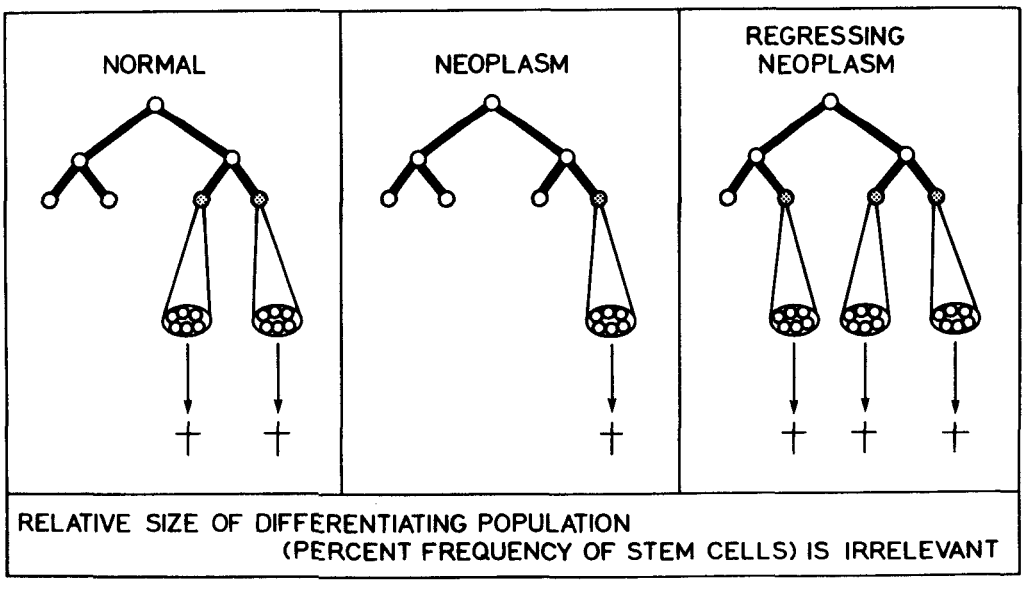



**Fig. 1.** Schematic diagram indicating that exponential increase in a cancer population occurs when more than 50% of stem cell progeny remain stem cells in nature. Regression of a cancer occurs when this percentage is reduced below 50%

row cells are essentially identical to those for induction of differentiation in leukemia colonies. Thus if the leukemic cells are responsive to differentiation induction, the quantitative aspects of their responsiveness to normal regulators are similar to those of responding normal cells (Table 1).

From the point of view of suppression of a leukemia population the induction of differentiation in even a high proportion of the leukemia cells is of relatively minor importance in determining whether or not the population expands in size progressively. The important question is whether the stem cells in the population continue to generate progeny, more than 50% of which remain stem cells in their proliferative potential. Regression of a leukemia population will only be achieved if the procedure used can force more than 50% of the progeny of stem cells to lose their stem cell properties, either by differentiation or death (Fig. 1).

This question seems not to have been addressed so far in studies of chemical inducers of differentiation, but in the case of biological regulators it has been demonstrated clearly that continuous culture of WEHI-3B cells in the presence of G-CSF (DF) leads to a marked and eventually complete suppression of stem cell self-replication [9]. This appears to be the basis on which DF suppresses the leukemogenicity of myeloid leukemia cells when tested by challenge injection into syngeneic recipients [5, 12].

## B. Early Events in Stem Cell Suppression

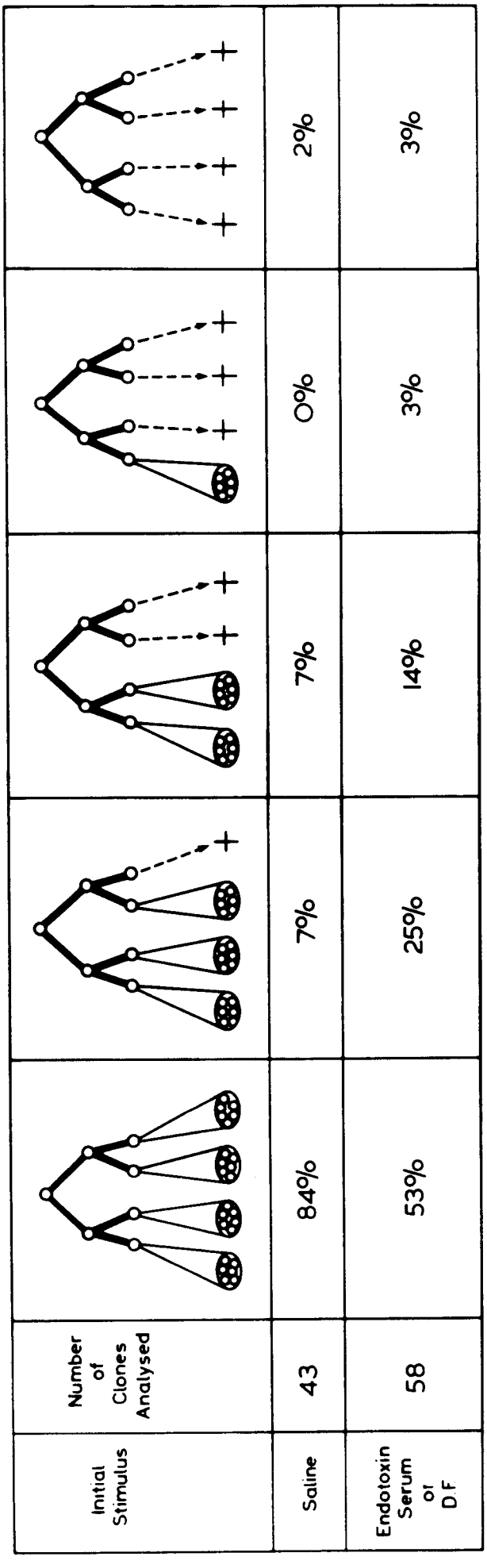
Because the leukemia cells forming colonies in semisolid agar cultures are demonstrable to be the stem cells in the leukemic population [14], analysis of the ability of leukemia colony-forming cells to self-generate (by recloning individual colony cells) is the most convenient method for monitoring leukemia stem cell self-generation.

Studies of this type have shown stem cell suppression usually to parallel the induction of differentiation in the same clone (colony) [8, 9], but have not determined whether one process precedes the other and whether the *continuous* presence of DF is required for maintenance of stem cells, as is true for the *proliferative* effects of CSFs on normal or leukemia cells.

In recently completed experiments these questions were answered by allowing WEHI-3B leukemia stem cells to divide once or twice in the presence of DF, then, by micromanipulation of washed progeny cells, determining by subsequent culture whether continuing clonogenicity (self-replicative capacity) of the daughter and granddaughter cells could be demonstrated if these cells were cultured subsequently in the absence of DF.

An example of the data obtained for granddaughter cells is shown in Fig. 2. It can be seen that exposure of the stem cells to DF for 18–22 h led to irreversible changes in many of the granddaughters of the stem cells, and in approximately half of the cases one or more of the granddaughters failed to produce a colony on recloning. Analysis of this suppression showed that the affected cell sometimes died without further division but more often divided once or twice in the succeeding 24-h period, after which the cells differentiated prematurely to polymorphs and then disintegrated [13].

These experiments clearly demonstrated that DF is not required continuously to suppress stem cell self-replication but is able to induce an irreversible change that continues to be expressed in the behavior of the progeny of such cells. This process was less evident in the analysis of daughter cells and conversely became more pronounced as clonal expansion increased to the 8–32 cell stage (44 h of incubation), where, for many clones, more than 50% of the stem cells in the clone were suppressed – a formal documentation of the validity of the theoretical proposition shown diagrammatically in Fig. 1 [13]. It will be noted in Fig. 2 that a marked asymmetry was evident in the behavior of the progeny cells formed in the presence of DF. Furthermore, because the cell cycle times of WEHI-3B stem cells are approximately 12 h, the data suggested that one full cell cycle in the presence of DF is sufficient to modify some of the progeny cells permanently. These observations raise the possibility that DF or a product of DF modifies one or more of the daughter chromatids in




the cell during S phase, possibly by influencing methylation or by insertion of some modulating or promoter sequence during synthesis of the daughter chromatids.

Complete suppression of all stem cells within a treated population appears to be achieved by attrition, with more and more of the progeny stem cells being modified as the exposure period continues.

Since there is clear evidence of heterogeneity in the responsiveness of individual stem cells to the induction of differentiation by DF, this "suppression by attrition" may be based on a similar heterogeneity in susceptibility to suppression. In its extreme form [the so-called differentiation-unresponsive state ($D^-$)], it has been demonstrated that co-incubation or prior incubation of $D^-$ cells with cytotoxic agents such as actinomycin D renders the cells responsive to the action of DF [4, 16]. Indeed, in at least some cases, the mechanism of induction of differentiation by chemical agents is likely to be indirect, the agents first provoking the leukemic cells themselves to synthesize DF, with subsequent autoinduction of differentiation [3].

## C. Sources of G-CSF

The existence of a special type of CSF with exceptional activity on leukemia cells (G-CSF or DF) was established unequivocally by studies on the serum from mice injected with endotoxin [2, 7]. Serum levels of G-CSF rise sharply following the injection of endotoxin and peak 3–6 h after injection [9]. Rises of serum G-CSF have also been observed in mice with acute infections [12].

A search of tissue sources for G-CSF revealed that many organs from normal or endotoxin-injected mice were able to synthesize G-CSF in vitro, in each case the

**Fig. 2.** Effect of presence of G-CSF (DF) during the first two cell divisions of WEHI-3B leukemic cells. Granddaughter cells were washed free of G-CSF (DF) and then individually recloned. Note that after exposure to G-CSF an increased percentage of granddaughter cells fail to generate colonies. In at least two-thirds of these instances suppression is asymmetric

molecule involved having similar properties and molecular weight [15]. It is still unclear which cell types can synthesize this molecule, but macrophages appear to be one cell with this capacity [13], whereas L cells (fibroblasts) appear to be inactive.

From many published in vitro studies on differentiation induction in cloned mouse myeloid leukemia cells, the impression is created that the critical question is whether the leukemia cells themselves produce, or can be induced to produce, G-CSF. This phenomenon has been documented to occur [3], and at least in the case of WEHI-3B cells the leukemia cells themselves continuously synthesize CSFs of a variety of types. In the context of a leukemia population in vivo, however, it is improbable that G-CSF produced by leukemia cells is a quantitatively important process, and it is more likely that G-CSF production by other tissues is of much more significance.

## D. Regulatory Factors Active on Human Myeloid Leukemia Cells

So far relatively little information is available on the existence and activity of naturally occurring regulatory factors for human myeloid leukemia cells. HL60 cells are clonogenic in semisolid agar culture and the colony-forming cells exhibit the capacity of self-replication [17]. It can be assumed therefore that by analogy with the WEHI-3B or M1 systems, the HL60 colony-forming cells are likely to be stem cells in the HL60 population. If this is so, comparable studies on the suppression of HL60 stem cells should be technically feasible using the same in vitro recloning assays used for WEHI-3B cells.

Addition of human placental conditioned medium (HPCM) to cultures of HL60 cells induces morphological changes in HL60 colonies comparable with those seen in differentiating WEHI-3B colonies, although the degree of differentiation induced is less striking than seen following the action of DF on WEHI-3B cells and resembles more closely the weaker action of GM-CSF on WEHI-3B cells (Table 2). This raises the possibility that although HPCM is known to contain two types of human-active GM-CSF (CSF$\alpha$ and CSF$\beta$), neither is analogous with murine G-CSF.

**Table 2.** Induction of differentiation in HL60 colonies by human placental conditioned medium (HPCM)

| Stimulus | Percent differentiated colonies | | |
|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 |
| Saline | 2 | 1 | 2 |
| HPCM 1:16 | 60 | 29 | 50 |
| HPCM 1:32 | 31 | 53 | 19 |
| HPCM 1:64 | 30 | 23 | 17 |
| Human urine | 3 | 9 | 2 |
| Mouse post-endotoxin Serum 1:6 | – | 0 | 0 |
| Mouse GM-CSF | 2 | 4 | 1 |

Replicate 2-ml agar cultures contained 1000 HL60 cells and 0.2 ml of the test material. Colonies were scored on day 14

In general, murine CSFs are inactive on normal human GM precursors and murine GM-CSF and DF preparations appear to be unable to induce differentiation in HL60 colonies. Human-active CSFs are active on normal mouse GM precursors, and it is of some interest that HPCM is an effective inducer of differentiation in WEHI-3B colonies (Table 3). Analysis of fractionated HPCM has suggested that CSF$\alpha$ is inactive but that CSF$\beta$ (the neutrophilic human active GM-CSF) is active on WEHI-3B cells. The implication from these studies is that at least some human GM-CSFs are active in enforcing differentiation of mouse myeloid leukemia cells. In this context it is of interest that some human sera, particularly from patients with active infections or severe neutropenia, are able to induce differentiation in WEHI-3B colonies [10].

So far as the studies have gone there seems to be little difference in principle between HL60 and mouse myeloid leukemia models, although human analogues of the highly active murine G-CSF have not yet been identified. Since G-CSF is elevated in the serum of mice with active infections, it is possible that elevated serum activity noted in patients with infections may be due to an analogous molecule, although human serum is not particularly

**Table 3.** Action of human placental conditioned medium in stimulating colony formation by normal mouse bone marrow cells and in inducing differentiation in mouse WEHI-3B leukemic colonies

| Stimulus | | Mean No. normal mouse bone marrow colonies | Mean percent differentiated WEHI-3B leukemic colonies |
|---|---|---|---|
| Saline | | 0 | 4 |
| Mouse post-endotoxin Serum | 1:6 | 100 | 97 |
| Human placental conditioned medium (HPCM) | 1:1 | 46 | 63 |
| | 1:4 | 32 | 40 |
| | 1:16 | 22 | 26 |
| | 1:64 | 0 | 19 |
| HPCM CSF$^{\alpha}$ | 1:1 | 28 | – |
| | 1:4 | 38 | 12 |
| | 1:16 | 19 | 11 |
| | 1:64 | 13 | 11 |
| HPCM CSF$^{\beta}$ | 1:1 | 43 | 91 |
| | 1:4 | 38 | 77 |
| | 1:16 | 25 | 29 |
| | 1:64 | 5 | 13 |

Duplicate assay cultures contained 75,000 C57BL marrow cells or 300 WEHI-3B leukemic cells and 0.1 ml of the test material. Colonies scored after 7 days of incubation

promising as a starting material for attempts to characterize biochemically the molecule active on human leukemic cells.

## E. Summary

Differentiation of mouse and human myeloid leukemic cells in vitro can be induced by some members of the granulocyte-macrophage family of colony-stimulating factors. In the mouse, the most active molecule (G-CSF) is able to suppress leukemic stem cell self-generation in an irreversible, asymmetric process, suggesting that the factor permanently modifies newly synthesized one or more daughter chromatids in dividing leukemic stem cells.

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