The Phenotypic Abnormality in Leukemia: A Defective Cell-Factor Interaction?

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Abstract

Differentiation of hemopoietic cells appears to depend upon specific interactions of certain cell-factors. The phenotypic abnormality in leukemia may involve an impairment in these interactions. In this report we present some of our views of leukemogenesis with respect to cell-factor interaction and the feasibility of experimental approaches to this problem. In culture, the interaction of myelogenous cells with factor(s) leading to differentiation can be measured either with a suspension mass culture method or by a solid (semi-soft) clonal method. The protein factors that support the growth of hemopoietic cells in suspension culture are termed growth stimulating factors (GSA) and in semi-solid culture, colony stimulating factors (CSA). Studies using conditioned medium prepared from phytohemagglutinin stimulated human lymphocytes (PHA-LyCM) and whole human embryo cells (WHE) revealed that GSA and CSA were not identical for growth of either normal human or leukemic leukocytes. In some cases maturation of leukemic leukocytes was observed. Fractionation of PHA-LyCM showed that there are three peaks for CSA. Each peak contains different fractions for supporting cellular proliferation, differentiation, and self-renewal of precursor cells in suspension culture. Apparently, each contains heterogenous species of protein factors some of which functionally overlap, while others do not.

Introduction

In order to carry out the normal function of hempoietic tissue, it is essential to have a continuous supply of mature functional cells, granulocytes, erythrocytes, megakaryocytes and plasma cells. These mature cells are derived from some progenitor cells which are committed for a particular pattern of differentiation, for example, granulocytes derived from a granulocytic cell progenitor (1, 2) and erythrocytes from an erythrocytic cell progenitor (3). The committed progenitor cells are in turn derived from some multipotent stem cells (4, 5, 6). The transition from precursor cells to differentiated cells requires some protein factors. Colony stimulating activity (CSA) and erythropoietin are examples of factors required for the transition from progenitor cells to differentiated cells (1–3). Little is known about

the factors required for the transition from stem cells to progenitor cells (7). Specific interactions between factors and factor responsive cells appears to be required for normal differentiation and proliferation. Disturbances in these interactions may impair the normal differentiation process. The phenotypic change in leukemia probably involves changes in the normal interaction between factors and the factor responsive cells, leading to a block in the normal maturation process (8, 9, 10). In this paper we wish to present our concepts of leukemogenesis with respect to cell-factor interactions and, more importantly, to present some preliminary results which illustrate the feasibility of experimental approaches to this problem.

Defectiveness of cell-factor interaction:

There are at least three means by which cell-factor interactions can be blocked (see Table 1). *First*, defectiveness in differentiation could be, and most likely is, in

Table 1: Defects in Cell-Factor Interactions

Defect	Response to factors *in vitro*
Factor responsive cells	–*
Factor	+
Factor producing cells	+

* Sometimes a positive response could be obtained either because of artificial conditions *in vitro* (there is less stringent control(s) *in vitro*) or because of the presence of some other factor for the specific abnormal situation.

the factor responsive cells. For example, if the membrane or factor receptor sites of the responsive cells is altered as a result of viral infection or treatment with physical or chemical agents, these cells may no longer respond to normal concentrations of factor (11). *Secondly*, defectiveness in differentiation could be due to the production of abnormal factors or to the presence of inhibitors. This situation would result in an insufficient concentration of factors for normal differentiation. *Thirdly*, the defect could be due to alteration(s) in the factor producing cells. For example, if the factor producing cells are infected by a virus, inadequate production of the factor might result. Among the three possibilities just described, the first is intrinsic to the factor responsive cells, while the other two are extrinsic to the responsive cells. Both the intrinsic and extrinsic causes may result in either an accumulation of early immature cells (such as in polycythemia vera and leukemia) and/or in a deficiency in the mature cell population (such as in neutropenia, aplastic anemia and pancytopenia).

The response of the factor responsive cells to exogenous factors in culture is different in each of the situations described (Table 1). In the first, most of the target cells cannot respond to factors, and therefore no growth and differentiation will be observed in culture. However, sometimes these defective cells might respond to factors under artificial *in vitro* conditions or respond to some specific factors. In the second and the third situations, one would predict that the factor

responsive cells will proliferate and differentiate in culture if proper exogenous factors are provided. In fact, results from many studies strongly suggest that *some* acute myelocytic leukemic cells from *some* patients grow and differentiate in culture while some apparently do not (12–16). This type of response or non-response has also been observed in some cases of neutropenia (17–19).

Measurement of factor-cell interactions:

Cell-factor interactions are expressed in three processes: proliferation, differentiation, and self-renewal. In granulocytic differentiation, these processes are conventionally measured in culture by two methods (Table 2). One is the colony

Table 2: Measurement of Cell-Factor Interaction

Methods	Factors Involved	Measurements/[1]
Semi-soft medium (agar or methylcellulose)	CSA	Colonies
Suspension culture	GSA	Cell counts and morphology
		[^{3}H]dThd
		Number of colony-forming cells

[1] The procedures for these measurements are described in the Legend to Figure 1.

forming assay in semi-soft agar (1, 2) or methylcellulose (20, 21). The factors involved in this assay are called colony stimulating activity (CSA) or colony stimulating factor (CSF). This method provides a quantitative measurement of the number of progenitor cells and also the differentiation and proliferation capacity of the progenitor cells (21, 22, 23). The other method is by suspension culture assay. By counting the cell number or by measuring thymidine uptake, it is possible to measure the proliferative capacity of the target cells. By observing the morphology of the cells, it is possible to determine the degree of differentiation. One can also measure the process of self-renewal by measuring the growth of colony-forming cells in suspension culture using the above mentioned colony technique in culture. The protein factors that stimulate these activities in a suspension culture are called growth stimulating activity (GSA). It is worth emphasizing that both CSA and GSA are defined according to their function as they might contain a pool of many activities. Identification of the protein factors corresponding to each functional entity is obviously critical in achieving an unambiguous understanding of the nature of these factor-cell interactions.

Contitioned medium from human lymphocytes:

Conditioned medium was prepared from normal human blood lymphocytes (24, 25). We have previously demonstrated that conditioned medium from this source is specific for human and subhuman primates, and it is relatively easy to prepare

(24). The procedure for preparation has previously been described in detail (24) but is also summarized here. 10^7 cells per ml are incubated in serum free Dulbecco's Modified Eagle's Medium for four to six days. When conditioned medium is prepared in the presence of phytohemagglutinin (PHA), the culture contains 10^6 lymphoid cells per ml, 1 % PHA-M (Difco), and 1 % homologous plasma in RPMI-1629 medium. The cells are incubated at 37 °C with 10 % CO_2. The medium is harvested the third day after incubation. The conditioned medium obtained in the presence of PHA (PHA-LyCM) contains CSA for human and monkey target cells but not mouse cells (Table 3 and Reference 24). Another advantage of

Table 3: Species Specificity and Colony Size from LCM and LyCM in the Presence and Absence of PHA

Source of Condition Medium	PHA	Colony Formation/[1]			Colony Size (Human Cells)
		Human	Primate	Mouse	
Leukocytes (LCM)	–	++		++	Medium to cluster/[2]
	+	++++		±	Large to medium
Lymphocyte (LyCM)	–	++		±	Medium to cluster
	+	++++	+++	±	Large to medium

[1] The procedure for the agar colony assay is described in the Legend to Figure 1.
[2] Cluster: less than 50 cells; small colony: about 50 to 200 cells; and large colony: more than 200 cells.

using PHA for factor production is that the size of colonies stimulated by this factor is much larger than those stimulated by conditioned medium without PHA stimulation. Very often the colonies contain several thousand cells. Conditioned medium prepared from buffy coat leukocytes in the presence of PHA are similar to those prepared from the lymphocyte enriched fraction with respect to species specificity and colony size (Table 3). In the studies described here, the experiments were mostly done with conditioned medium prepared from lymphocyte-enriched fraction in the presence of PHA. We found that CSA is produced both from B and T lymphocyte populations. While the B lymphocyte fraction is able to produce CSA without PHA stimulation, the T cell population requires PHA stimulation. It has been reported that CSA for human cells are produced from human monocytes (26, 27) or adherent cells (28). Our findings would suggest that CSA are produced from more than one type of cell. Alternatively, the factor producing cells may be co-fractionated with both adherent and non-adherent cells.

CSA activity in PHA-LyCM:

As discussed above, PHA-LyCM contained CSA that stimulates the formation of large size colonies in semi-soft agar medium. Six types of colonies can be distinguished by morphology (Wu, Glick and Gallo, manuscript in preparation). Some have been described before (21, 23). Type-1 colonies are compact with un-

even rigid edges. They contain mainly eosinophilic granulocytes which are peroxidase positive. Type-2 colonies look compact in the center with cells loosely distributed in the periphery. This type of colony consists mainly of neutrophilic granulocytes or monocytes which are peroxidase positive. Type-3 colonies are those with cells evenly and loosely distributed. These are peroxidase negative macrophage cells. Type-4 are colonies that are scattered with clumps of cells. Each clump contains less than 10 cells. Each cell in the clump is relatively large compared with other colony cells. It will be of interest to determine if these cells are megakaryocytes. Type-5 colonies are the clusters which contain less than 50 cells. They could look like type-1 or type-2 except for their smaller cell numbers. Finally, type-6 colonies are those which contain an aggregation of several clusters (less than 50 cells per cluster). This type of colony resembles the "burst" formation in erythrocytic colony formation in the plasma clot system (34). This type of colony sometimes is difficult to distinguish betwenn single-cell origin or multi-cell origin. At the conclusion of morphological identification, one should point out that the correlation between colony morphology and histochemical properties of colony cells is a relatively rough one. For precise study, the identification of the nature of colonies still requires histochemical staining.

The CSA described here are relatively heat stable. They are active after treatment at 70 °C for one hour (Table 4). After treatment at 90 °C for an

Table 4: Some Properties of LyCM

Treatment/[1]	Colonies/10^5 Cells/[2]
None (37 °C)	41
56 °C	50
70 °C	49
90 °C	0
Trypsin (100 µg/ml)	0
Neuraminidase (100 µg/ml)	0
0.5 M NaCl	0

[1] LyCM are treated at 37 °C, 56 °C, 70 °C and 90 °C respectively for one hour. Trypsin treatment was performed at 37 °C for one hour. Neuraminidase treatment was performed at 37 °C for one hour.
[2] Average of 4 plates.

hour, all type-1, 2, 3, and 5 colonies disappear, but type-4 and 6 colonies are not significantly affected. This suggests that there are different types of CSA with respect to heat sensitivity. All CSA are sensitive to trypsin and neurominidase treatment, suggesting that CSA are glycoproteins. Another feature of CSA from PHA-LyCM is that they are sensitive to high salt treatment. The activity can be completely restored after removal of the salt by a dialysis against phosphate buffered saline (PBS), but dilution of the high salt treated CSA with PBS is not sufficient to restore the activity. This observation suggests that an active form of CSA is formed during the dialysis, for example, the formation of a polymer.

GSA in PHA-LyCM

GSA in PHA-LyCM are measured by three procedures in suspension culture, namely, viable cell number, [^{3}H] thymidine uptake and number of CFC following culture. The suspension culture technique is similar to that described by Aye *et al.* (29). Three ml of cell suspension containing 20 % fetal calf serum in medium 10^6 nucleated cells and 20 % of PHA-LyCM is placed in a T30 plastic flask. When PHA-LyCM is omitted, an equivalent amount of PHA is included in the culture medium. The number of viable cells is estimated by the trypan blue exclusion technique. [^{3}H] thymidine uptake is performed by labelling cells with 5 μc/

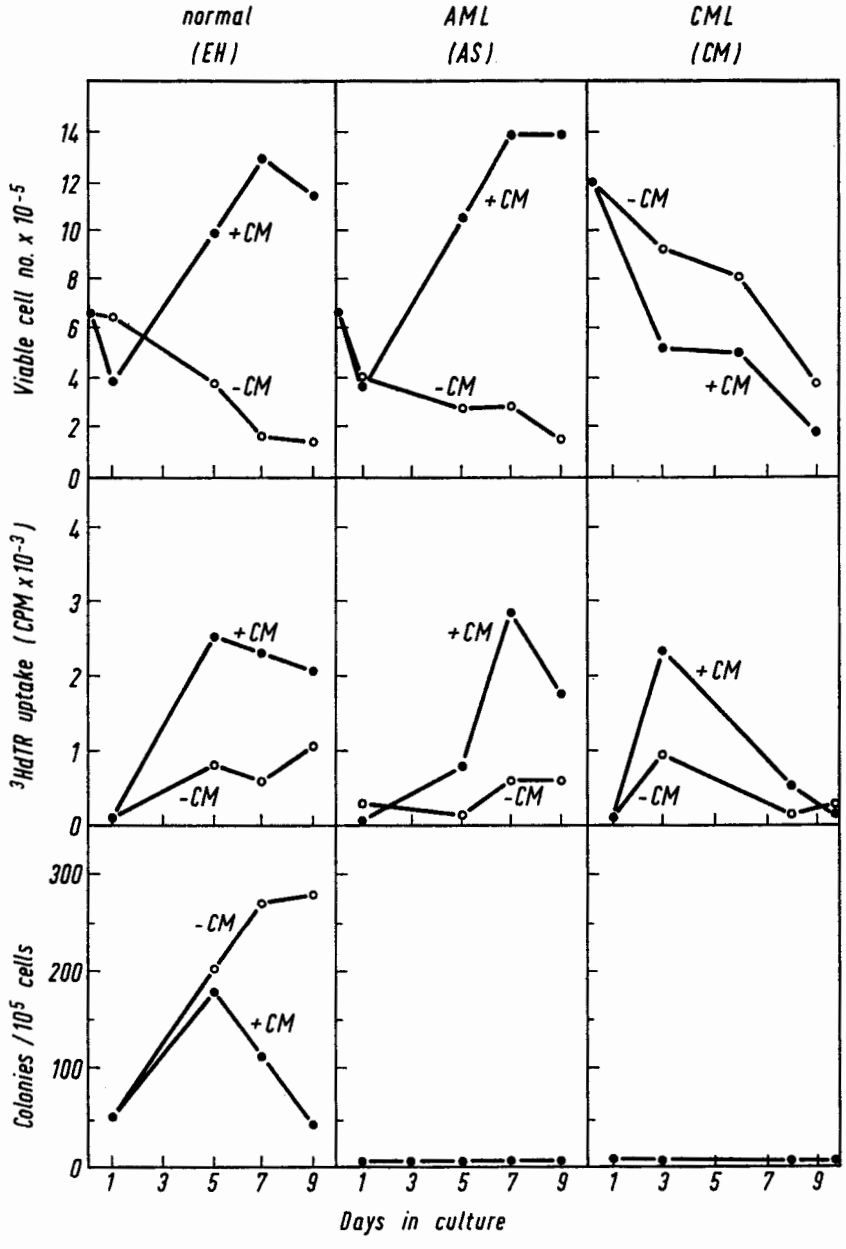

Fig. 1: *Growth of Normal and Leukemic Leukocytes in Suspension Culture.*
Non-adherent cells (NAC) are prepared according to the procedures described by Messner *et al.* (28). Bone marrow cells from a normal woman (EH), a woman with acute myelogenous leukemia (AML), patient (A. S.), and a woman with chronic myelogenous leukemia (CML), patient (OM), were separately placed on petri dishes (Falcon plastic) and incubated

at 37 °C with 10 % CO_2 for one hour. Those cells that did not adhere to the dishes were decanted onto an dish and the incubations were repeated for 2½ hours. These NAC were then used for all of the experiments. The suspension culture contained 1.6 x 10^6 nucleated cells/ml, 60 % α medium, 20 % fetal calf serum, and 20 % LyCM. Since LyCM contained 1 % PHA-M, the 1 % PHA-M was used for control culture without LyCM. Three ml of this culture were placed in a T60 flask and were incubated at 37 °C with 10 % CO_2. Samples were harvested at different days and viable cell number, [^{3}H]dThd uptake, and number of colony forming cells were measured. Viable cell count was performed with a standard trypan blue exclusion technique. For [^{3}H]dThd uptake, 15 μc of [^{3}H]dThd were added to 3 ml of suspension culture for four hours before the harvest of cells. The harvested cells were washed twice with phosphate buffered saline (PBS) and twice with high pH buffer (0.01 M Tris; pH 8.8, 0.14 M NaCl, 0.02 M $MgCl_2$) at 4 °C. The cell pellet was then lysed with 1 ml of the same buffer containing 1 % Triton X-100. Aliquots (50 and 100 μl) of lysate were precipitated with 10 % trichloroacetic acid (TCA) at 4 °C. The acid precipitable count was the determined. The number of colony forming cells was measured by an agar colony assay according to the procedures we have previously described (24). Briefly, the assay consisted of two layers of soft agar medium in a 60 mm petri dish. The upper layer contained 0.8 ml of 0.3 % agar in McCoy's 5A medium containing 20 % fetal calf serum and 10^5 nucleated NAC, and the lower layer 2.5 ml of 0.5 % agar in McCoy's 5A medium containing 20 % fetal calf serum and 25 % LyCM. Cultures were incubated at 37 °C with 10 % CO_2. Colonies were examined between 12 to 14 days after incubation. When more than 50 cells were found, they were scored as positives as a colony.

ml of [^{3}H] thymidine for 4 hours. The number of CFC in suspension culture is measured by plating 10^5 viable cells in an upper layer of agar medium in a 60 mm petri dish containing 20 % of PHA-LyCM in the lower layer of the petri dish. Under these conditions, colony formation is still dependent on CSA, suggesting that CSA is still required for colony formation although these cells were exposed to CSA in suspension culture.

Figure 1 shows that the growth of non-adherent cells from normal human bone marrow cells is stimulated by the PHA-LyCM measured both by viable cell count and by DNA synthesis. However, CFC from normal non-adherent cells increased even in the absence of PHA-LyCM. This GSA independent self-renewal process is inhibited by PHA-LyCM in the later period of the culture. We observed that when unfractionated or adherent marrow cells were used for this study, no stimulation of cell growth was observed. Results from assays of bone marrow cells from a patient with AML and one from CML are also shown in Figure 1. PHA-LyCM produces an exponential increase in the number of viable cells in the case of AML while there is no detectable effect on the cell number in the case of CML, although DNA synthesis is stimulated in both. Cells from both are not able to replicate CFC nor respond to stimulation with conditioned medium. These results clearly demonstrate that PHA-LyCM contains GSA and that these GSA are heterogenous with respect to the three activities measured in this study. The GSA responsible for each activity appear to be separable, although it is still questionable whether PHA-LyCM contains activity sufficient for self-renewal. However, we would like to emphasize that these results are given to stress the heterogeneity among GSA and CSA and not to be generalized as a specific pattern of response of a specific leukemia to PHA-LyCM.

CSA and GSA are not identical:

Protein species may exist which contain overlapping CSA and GSA, but the major protein sources of these activities are clearly not identical. For example, as illustrated in Table 5, factors A, B, C, D, and E are required for the formation

Table 5: Model of Relationship Between CSA and GSA

activity	*protein species**
CSA	A. B. C. D. E.
GSA	C. D. E. F. G.

* The number and name of protein species are hypothetical.

of one type of colony in a semi-soft agar medium, while factors C, D, E, F, and G are required for the growth and maturation of hemopoietic cells in suspension culture. Factors C, D, and E are required for both assays but factor A, B, F, or G are required only for one particular assay. Some observations shown in Table 6 illustrate this point. Bone marrow cells from a patient with AML (AS) are grown in suspension culture containing either PHA-LyCM or WHE-1 CM (conditioned medium prepared from a particular cell line of whole human embryo),

Table 6: Some Results which Differentiate CSA and GSA

Source of Factor Responsive Cells	Contitioned Medium	Colony Formation in Semi-Soft Agar Medium/[3]	Proliferation in Suspension Culture/[4]
AML (AS)	none	–	–
	LyCM	–	+++
	WHE-1 CM/[2]	–	+++
AMML (JC)	none	–	+++
NA/[1]	LyCM	+++	±
	WHE-1 CM	+++	+
Normal (EH)	none	+	–
NA	LyCM	+++	++
	WHE-1 CM	+	–

[1] NA: non-adherent cells. These are prepared according to the procedure of Messner *et al.* (28).

[2] WHE-1 CM denotes conditioned medium prepared from whole human embryo cell strain no. 1 in our laboratory. The conditioned medium was harvested 48 hours after WHE-1 cells reached stationary phase.

[3] The procedures for the agar colony assay are described in the Legend to Figure 1. – = no colony formation; + = one colony per 10^4 nucleated cells; +++ = one colony per 10^3 nucleated cells.

[4] This is based on results obtained from viable cell count. – = no stimulation of cell proliferation; + = slight stimulation of cell growth; ++ = moderate stimulation of cell growth; +++ = exponential growth of the factor responsive cells.

but the same cells do not form any agar colonies in the presence of either factor. Conversely, marrow cells from a case of AMML (acute monomyelocyte leukemia) (JC) form agar colonies in the presence of the conditioned medium, but no growth was observed in the suspension culture, although the growth of these cells is observed in the absence of exogenous conditioned medium. It is likely that these leukemic cells produced some unique protein factors that support the growth of leukemic cells, and these factors are ineffective in the presence of protein factors isolated from normal cells. Normal marrow cells grow both in agar medium culture and suspension culture in the presence of PHA-LyCM, but WHE-1 CM supports the growth of normal cells in agar medium culture but not in suspension culture. Apparently, WHE-1 CM is able to support the growth of some leukemic cells in suspension culture in an exponential manner but not normal cells. In this aspect, this is similar to the factor(s) described by Gallagher *et al.* (30). These results not only show that CSA and GSA are not identical, but also that the ability of GSA in supporting the growth of normal and some leukemic leukocytes could be different.

Fractionation of PHA-LyCM:

An attempt was made to separate various activities in PHA-LyCM. Conditioned medium was fractionated by conventional biochemical procedures, including ammonium sulfate precipitation, sephadex gel filtration (G200) and DEAE cellulose chromatography. Table 7 summarizes the result of this fractionation pro-

Table 7: Summary of Purification of PHA-CSF

Purification step/[1]	Volume (ml)	Activity/[2] (units/ml)	Protein (mg/ml)	Specific Activity (units/mg)	Purification	Yield
I. Unpurified	15000	120	0.78	153	1	
II. ASP (45–65 %)/[3]	200	18000	100	280	1.8	100
III. Sephadex G 200						
Peak I	460	2000	1.53	1310	8.5	16.4
Peak II	640	6300	0.48	13120	85.8	71.4
IV. DEAE Cellulose						
Peak I	380	4900	0.76	6400	42.1	33.9
Peak II	225	10400	0.60	17600	113	41.1
Peak III	175	9600	0.36	26700	175	30.4

[1] The details of purification procedures will be described elsewhere (31).
[2] Unit is defined as colony number per 10^5 cells stimulated by 1 % increment of CSF (in a linear region).
[3] Inhibitory activity is detected in the ammonium sulfate precipitate (ASP) (45–65 %).

cedure; the details of which will be described elsewhere (31). After DEAE cellulose chromatography, three peaks of CSA are obtained with estimated molecular weights of 70,000, 40,000 and 25,000 respectively. Peak I predominantly stimulates type-3 colony growth, and Peak II and III stimulate all types of colonies. An attempt was made to distinguish their colony stimulatory ability, but clear results were not obtaned probably because Peaks II and III were not well separated. The GSA in each CSA peak were measured with normal cells and are shown in Table 8. Peak I sti-

Table 8: GSA in Purified CSA Preparation

Factors [1]	Suspension Culture/[2] (normal marrow cells)			Semi-soft/[3] Agar Medium Colony Formation (units x 10^{-3}/ml)
	Viable Count (x 10^{-6} cell/ml)	[^{3}H]dThd Uptake (cpm x 10^{-3})	CFC in Culture (colonies/10^5 cells)	
None	2.3	2.6	105	0
D I	2.1	5.0	105	4.9
D II	2.2	2.8	165	10.4
D III	2.2	4.5	125	9.6

[1] DI, DII and DIII represent Peak I, II and III CSA after DEAE cellulose chromatography shown in Table 7. For all experiments only 2 % of each fraction was used.
[2] The procedures for the suspension culture are described in the Legend to Figure 1.
[3] These results were the same as those shown in Table 7.

mulates [^{3}H] thymidine uptake, but it does not enhance the number of viable cells nor of CFC. Peak II enhances the growth of CFC but not of viable cells nor [^{3}H] thymidine uptake. Peak III is similar to Peak I in that only [^{3}H] thymidine uptake is enhanced in the suspension culture. Although these results are preliminary, they suggest that it is feasible to study a specific interaction between protein factors and their specific target cells with fractionated defined factors. We are currently looking for some peak activities containing the self-renewal activity. Success in the isolation of this activity will be important for establishing measurements of multipotent stem cells in culture.

Conclusions

We believe that a generalized statement regarding leukemia as a stem cell disease is unwarranted. It is more likely that stem cells are one cell type that can be transformed. Other cell types may also be target cells, but the term leukemic transformation should not be loosely used since it is difficult and sometimes impossible to distinguish between the accumulation of normal immature cells and the accumulation of abnormal blood cells. Elucidation of cell-factor interactions should be very useful in making the distinction.

To study specific cell-factor interactions, well-defined quantitative assays are required. At present, the literature concerning CSA and GSA for myeloid cell dif-

ferentiation describe many activities which are ill-defined. Even the growth of erythroid colonies in culture stimulated by erythropoietin (3, 32, 33) are not well defined. To obtain quantitative measurements of each cell-factor interaction we need: 1) fractionation and purification of protein factors that promote the growth and differentiation of hemopoietic cells; 2) determination of the interrelationship among various factors; 3) fractionation and identification of target cells for specific protein factors; and 4) identification of specific biochemical events in cells associated with a particular cell-factor interaction. The studies presented in this paper represent some of our initial attempts in pursuing these goals.

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